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The role of PRH/HHEX in TGF- signaling and cancer cell-platelet interactions

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MSC BY RESEARCH DISSERTATION

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Abbreviations

ALK5 – Activin-like kinase 5

BPH – Benign prostatic hyperplasia

CM – Complete media

coSMAD – Common partner SMAD

EMT – Epithelial-to-mesenchymal transition

FBS – Fetal bovine serum

NK – Natural Killer

PRH – Proline-rich homeodomain protein

pPRH – Phospho-PRH

qPCR – Quantitative PCR

SD – Standard deviation

SDS-PAGE – Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SMAD – Mothers against decapentaplegic

TGF-βR – TGF-β receptor

TGF-β – Transforming growth factor β

TLE – Transducin-like enhancer

VEGFA – Vascular endothelial growth factor A

Figures

Acknowledgements

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Abstract

Dysregulated TGF-β signalling is implicated in supporting metastasis of cancer cells by promoting an epithelial-to-mesenchymal transition (EMT), with blood platelets proposed to represent a rich source of TGF-β for cancer cells that migrate into the vasculature. The proline-rich homeodomain protein (PRH) is a transcription factor that inhibits EMT and cell migration in part at least through modulation of TGF-β signalling. PRH levels are post-transcriptionally regulated via phosphorylation by the protein kinase CK2, which leads to subsequent proteasomal cleavage. PRH is hyper-phosphorylated in prostate cancer tissues and cell lines due to aberrant CK2 activity, potentially removing a restriction on pro-EMT TGF-β signalling. In the following MSc by Research project preliminary data is presented which suggests that TGF-β may promote a decrease in PRH abundance in 'normal' prostate PNT2-C2 cells, but by contrast may cause an increase in PRH levels in PC3 prostate cancer cells. Additionally, it was observed that in PC3 cells TGF-β may increase the abundance of phosphorylated PRH in a CK2-independent mechanism. It was found that platelet treatment did not recapitulate these findings, although preliminary observations suggest there may be changes at the mRNA level in the timeframe studied. Finally, it was observed that overexpression of PRH blocked TGF-β-mediated EMT changes, suggesting a possible target for prevention of pathological EMT in the body.

Introduction

Prostate cancer

In 2012, prostate cancer was attributed to around 300,000 deaths worldwide, making it the fifth leading cause of cancer death [1]. There were over 1,000,000 new diagnoses in 2012, making it the second-most diagnosed cancer in men globally. In developed countries, it is the most common cancer among men. Although prostate cancer deaths are decreasing across most developed countries, they are rising in regions such as Eastern Europe, Russia and Asia, compounding the need to develop better preventative and therapeutic strategies.

Metastasis

Metastasis is the spread of cancer cells from the primary tumour to distant sites around the body, and corresponds to poor prognosis. Prostate cancers, for example, often metastasise to bone, where they can disrupt haematopoiesis or stimulate bone overgrowth leading to pain, fractures or spinal cord compression [2]. Genetic alterations promoting metastatic behaviour can confer drug resistance [3], while some organ microenvironments may provide inherent protection against therapy [4]. Metastasis represents a sequence of physiological events, as a cancer cell must leave the primary tumour, invade local tissue, enter the bloodstream, disseminate, leave the vasculature and colonise a distant organ. The epithelial-to-mesenchymal transition (EMT) has been widely implicated in initiating and promoting this metastatic cascade [5,6]. Epithelial cells exist as apico-basal polarized sheets, stabilised laterally by cell-cell adhesion and anchorage to the basement membrane. EMT involves the loss of basolateral adhesion and the acquisition of a more elongated, motile phenotype. Accordingly, EMT is characterised by the loss of E-cadherin and other adherence proteins and the concomitant upregulation of mesenchymal markers such as vimentin, N-cadherin and the secretion of matrix metalloproteases (**Fig. 1**) [5]. EMT is crucial for many developmental processes in the embryo, including gastrulation and neural crest development. However, cancer metastasis may be promoted by inappropriate EMT, enabling cells to remodel the extracellular matrix, leave the parenchyma of a primary tumour and enter the vasculature, resulting in dissemination.

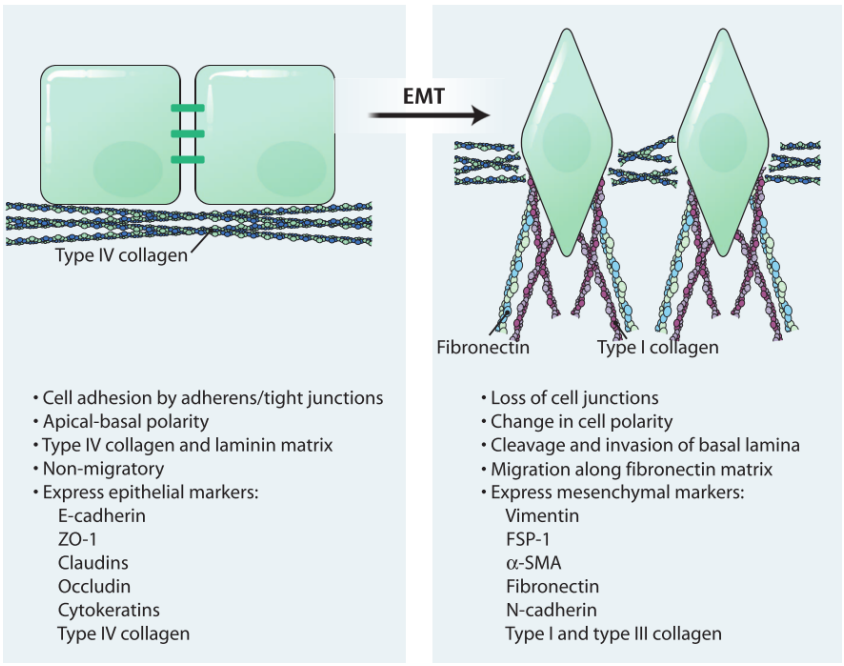


Figure 1. Changes in cell adhesion, polarity and morphology associated with EMT

Epithelial cells possess apico-basal polarity and exhibit lateral cell-cell adhesion mediated by molecules including claudins, zona occludens 1 (ZO-1) and E-cadherin. These cells are associated with a basal matrix predeominantly composed of laminin and type IV collagen (**left**). During EMT, cells lose epithelial adherence proteins with concomitant increase in mesenchymal markers such as vimentin, fibroblast specific protein 1 (FSP-1), α-smooth muscle actin (α-SMA) and N-cadherin. Mesenchymal cells acquire a spindle-shaped morphology and invade the surrounding extracellular matrix, migrating along a secreted fibronectin matrix (**right**) [5].

TGF-β signalling

Inappropriate EMT may be prompted by aberrant transforming growth factor β (TGF-β) signalling. The TGF-β superfamily consists of 33 cytokines, including 3 isoforms of TGF-β, which initiate signalling via the formation of a heterotetrameric complex composed of type I and type II TGF-β receptors. Varying combinations of these receptors convey differential ligand binding, thereby enabling different responses to the same cytokine [7]. Upon ligand-mediated oligomerisation, the constitutively active type II receptor phosphorylates the type I receptor (TGF-βRII and TGF-βRI, respectively), creating a docking site for receptor-SMAD (mothers against decapentaplegic) proteins, SMAD2/3. The receptor-SMADs are the cytoplasmic effector proteins of the canonical TGF-β signalling pathway, and their activity is tightly regulated via post-transcriptional modifications including phosphorylation and ubiquitination [8–10]. Once SMAD2/3 are C-terminally phosphorylated by TGF-βRI they associate with the common partner (co-SMAD) SMAD4, and the activated SMAD trimer translocates to the nucleus (**Fig. 2**). Here, the complex may bind in the minor groove of the DNA helix to regulate gene expression and epigenetic processes in cooperation with various other factors [11]. TGF-β may also directly or indirectly induce other signalling pathways, such as the Erk MAP kinase pathway [12]. TGF-β signalling is involved in coordinating EMT in the developing embryo but in the adult TGF-β signalling performs numerous homeostatic roles, typically acting as a suppressor of cell proliferation [13–15]. This anti-proliferation signalling can become inactivated in cancer cells through a number of mechanisms, causing these cells to respond to TGF-β with only pro-EMT signalling instead [16,17]. For example, normal p53 complexes with SMAD proteins to enact TGF-β-mediated cytostasis (inhibition of cell growth and division) but mutant p53 in this complex instead binds and sequesters p63, preventing p63 from antagonising EMT signalling [18]. TGF-β, primarily through the TGF-βRI activin-like kinase 5 (ALK5), ultimately promotes EMT by inducing the expression and activation of a number of EMT transcription factors. Snail and Slug, for example, are EMT transcription factors that repress transcription from *CDH1* encoding E-cadherin, a key marker for EMT (**Fig. 2**) [19]. Twist1, another transcription factor up-regulated by TGF-β signalling, up-regulates Snail expression and also complexes with BMI1 to repress E-cadherin and p16-INK4a, a cell cycle inhibitor [20,21]. EMT signalling also upregulates the ubiquitin ligase HDM2 which degrades the tumour suppressor p53, inhibiting the action of specific miRNAs that would moderate EMT signalling [22]. Through these mechanisms, TGF-β signalling can promote an EMT that facilitates motility and metastasis of cancer cells.

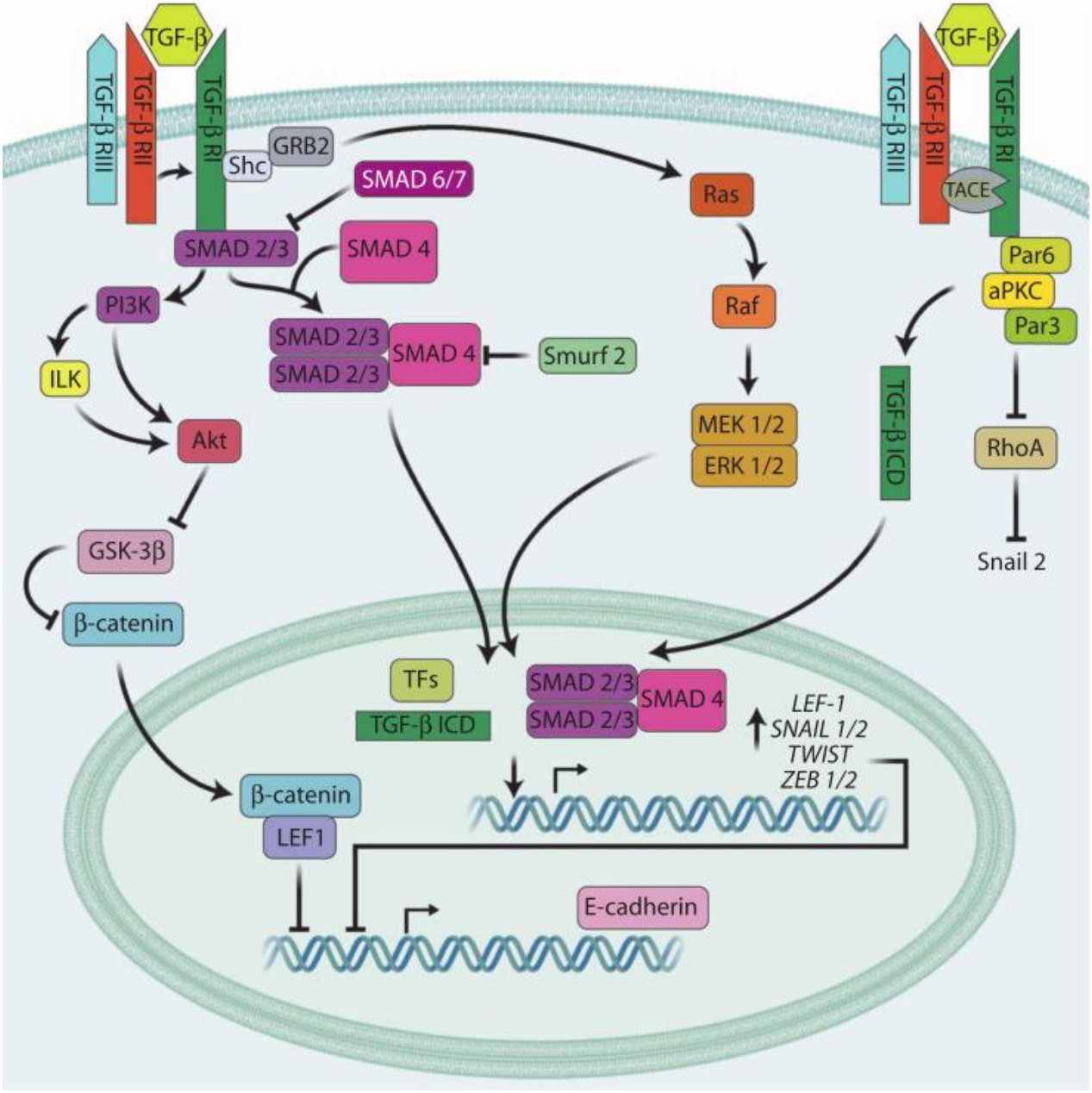


Figure 2. Regulation of EMT by TGF-β

Figure 2. Regulation of EMT by TGF-β

Ligand-mediated oligomerisation of TGF-β receptors results in phosphorylation of TGF-βRI and subsequent activation of numerous signalling pathways, including those facilitated by SMAD2/3, Ras and PI3K. SMAD2/3 is phosphorylated by TGF-βRI and associates with SMAD4 to form a trimer, which then translocates to the nucleus along with other factors to induce expression of pro-EMT transcription factors such as Snail1/2 and Twist1. Additionally, TACE may cleave TGF-βRI at the cell membrane to release its intracellular domain, which may act to regulate EMT. Also at the cell surface, the Par3-Par6-atypical PKC complex interacts with TGF-βRI and promotes cytoskeletal remodelling associated with acquiring a mesenchymal phenotype [5].

Platelets and metastasis

Blood platelets have long been implicated in promoting metastasis, since inhibition of coagulation reduces metastasis in several experimental models [23,24]. Some cancer cells express selectin ligands, which are bound by P-selectin on platelets. The resulting platelet adhesion protects cancer cells from shearing in the vasculature [25], and can shield them from natural killer (NK) cells both physically and through signalling leading to quiescence in NK cells [26]. Further, some cancer cells may activate platelets via expression of tissue factor, a transmembrane receptor that complexes with the bloodborne Factor VII/VIIa to initiate the coagulation cascade [27]. Upon activation, platelets can support tumour growth and angiogenesis by releasing a variety of factors, including TGF-β (**Fig. 3**). Indeed, platelets represent the most prominent store of TGF-β in the body, containing 40-100-fold more of the cytokine than other cell types [28,29]. Incubation with platelets has been shown to activate the TGF-β/SMAD pathway in breast and colon carcinoma cells, induce EMT and promote metastasis in mice [30]. Pre-treating cells with platelets or TGF-β prior to implantation fails to enhance metastasis in mice with TGF-β1-deficient platelets, highlighting the importance of platelet-derived TGF-β in this process. Furthermore, platelets induce greater pro-metastatic changes in gene expression in cancer cells than purified TGF-β alone via a contact-dependent, synergistic activation of the NFκB pathway [30]. In this manner platelets may meet metastatic cancer cells in the bloodstream and reinforce their mesenchymal phenotype, thereby facilitating extravasation and migration to a secondary niche.

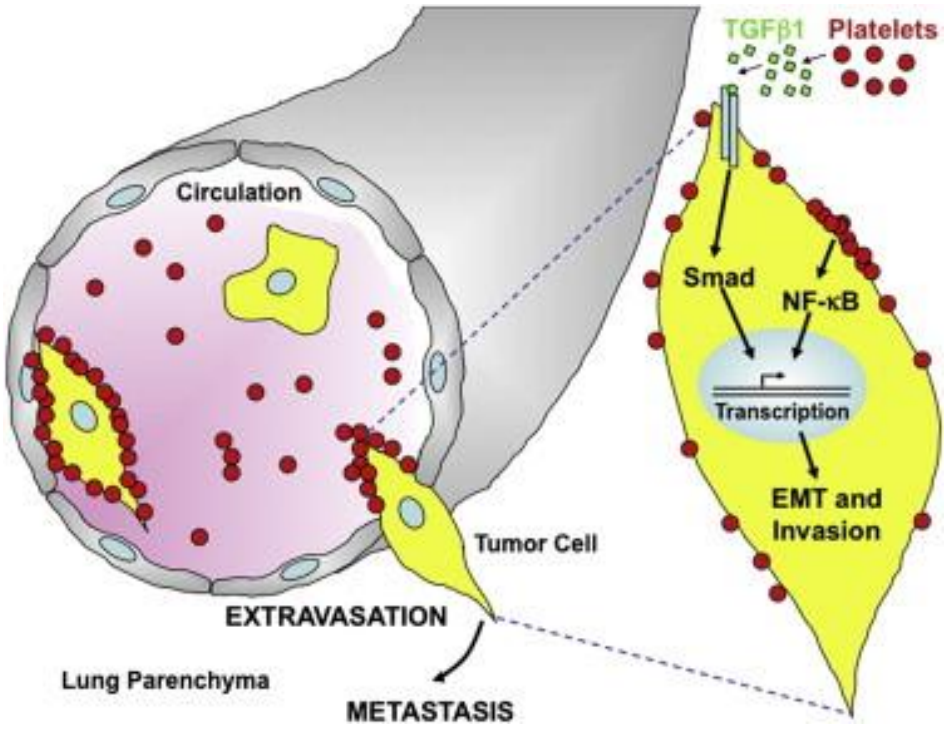


Figure 3. Platelets support EMT through direct contact and TGF-β release

Figure 3. Platelets support EMT through direct contact and TGF-β release

Cancer cells that have migrated into the bloodstream may bind and activate platelets via the expression of cell surface selectins. Activated platelets can secrete TGF- β and activate NF-κB signalling in contact-dependent signalling, with synergistic activation of both pathways acting to promote EMT in cancer cells. Furthermore, platelets may provide protection from mechanical stresses and NK cell killing in the vasculature. Reinforcement of EMT in the bloodstream may better enable cancer cells to extravasate and migrate to a supportive secondary niche, thus promoting metastasis [30].

PRH regulates many processes via several mechanisms

The proline rich homeodomain protein (PRH) is an oligomeric transcription factor expressed in a range of tissues in the embryo and the adult [31,32]. It has a wide variety of genomic targets, with several roles in development and its dysfunction is associated with various disease states and cancers, including liver, breast, prostate and thyroid cancers [33–36]. Depending on the target gene, PRH can either repress or activate transcription (**Fig. 4, left**). PRH oligomerises through an interaction between its N-terminal domain and homeodomain and binds tandem arrays of its DNA consensus sequence to condense DNA. Repression is achieved via concomitant recruitment of Groucho/TLE (transducin-like enhancer) co-repressors using the N-terminal domain [37]. In addition, it was recently shown that PRH can recruit Polycomb group proteins, resulting in epigenetic silencing of target genes [38]. PRH can also occlude TATA boxes within promotor sequences, and can interact with transcription factors in order to sequester them [39]. Repressed genes include the pro-EMT transcription factor Goosecoid and vascular endothelial growth factor A (VEGFA) [40,41]. Transcriptional activation is likely achieved via direct interactions with its C-terminal domain. PRH binds directly to promotor regions of the genes it activates, such as the Na(+)-bile acid cotransporter gene [42]. It can also act as a co-activator in association with other factors [43]. Additionally, PRH binds eIF4E within promyelocytic leukaemia protein bodies, preventing the nuclear export of some transcripts. One such mRNA is Cyclin D1, thereby inhibiting progression of the cell cycle [44]. PRH can also limit cell proliferation through blocking the DNA binding activity of c-Myc, by disrupting its interaction with its obligate partner Max [45]. Thus, PRH dysfunction is potentially tumourigenic through the derepression of VEGF signalling, Goosecoid expression and c-Myc DNA binding.

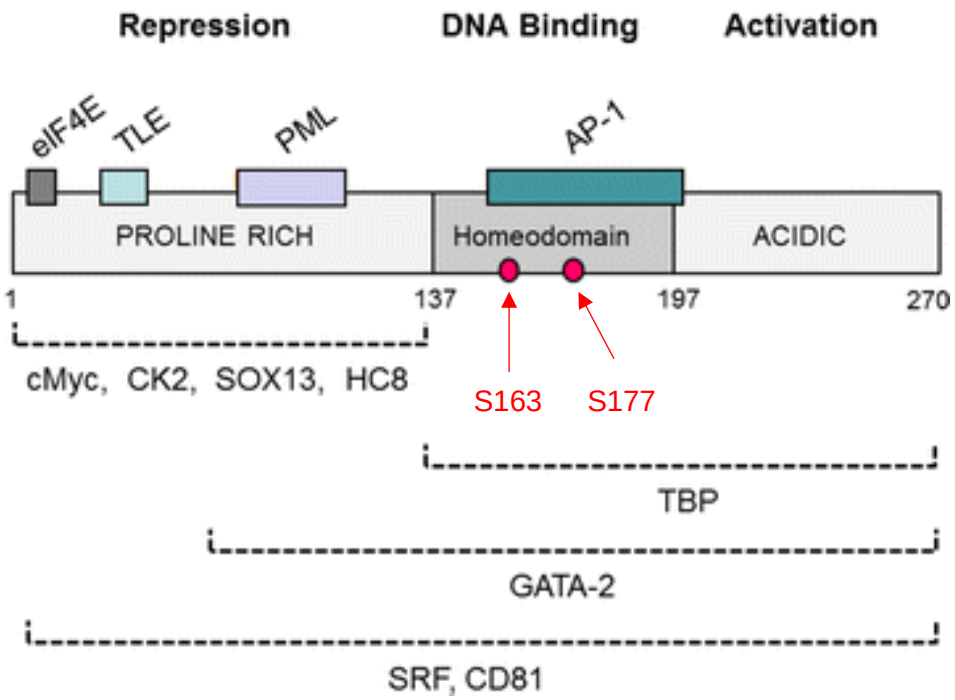


Figure 4. The domain structure of PRH and its modulation of TGF-β signalling

Figure 4. The domain structure of PRH and its modulation of TGF-β signalling

PRH is a 270 amino acid protein composed of a 136 amino acid N-terminal domain involved in transcriptional repression, a central 60 amino acid homeodomain that mediates sequence-specific DNA binding, and a 73 amino acid C-terminal domain required for transcriptional activation. Oligomerisation is facilitated by interactions between the N-terminal domain and the DNA binding domain, enabling PRH to bind tandem repeats of its consensus sequence. Coloured boxes represent regions of PRH known to interact with the indicated proteins. The dashed brackets represent interactions that are known but currently less well defined. Phosphorylation sites within the DNA binding domain, dictating binding ability, are highlighted in red. Adapted from [43].

Endoglin

One gene known to be positively regulated by PRH is Endoglin [36]. This gene encodes a type III TGF-β receptor that acts as an auxiliary factor to modulate TGF-β signalling [46]. The endoglin protein lacks catalytic activity but contains Ser/Thr residues that can be phosphorylated by both ALK5 and TGF-βRII. This is followed by dissociation of ALK5 from the complex and cessation of TGF-β signalling [47]. Endoglin also suppresses cell motility via signalling through the ALK2-SMAD1 pathway [48]. While Endoglin is both up and down-regulated in different cancers, it has been demonstrated to be down-regulated in prostate cancer cell lines, and its expression correlates inversely with cancer malignancy and invasiveness [46]. Loss of Endoglin is therefore potentially pro-metastatic as it allows TGF-β-ALK5 pro-motility signalling to function unopposed, promoting inappropriate EMT and cell motility. Importantly, it has been demonstrated that PRH inhibits cell migration in breast and prostate cells via its regulation of the Endoglin gene, and that loss of PRH induces a migratory phenotype (**Fig. 5**) [36].

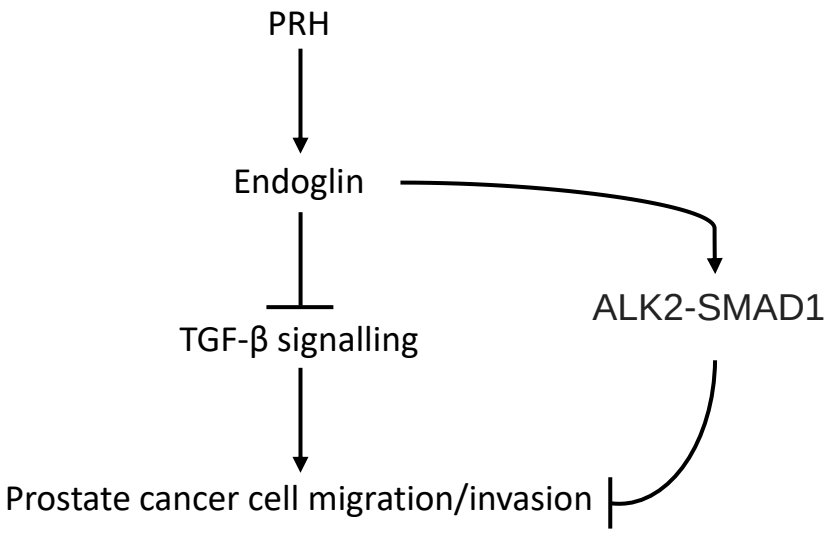


Figure 5. PRH modulates TGF-β signalling via its regulation of Endoglin

PRH directly upregulates expression of Endoglin, an auxiliary type III TGF-β receptor. At the cell membrane Endoglin associates with ALK5 and TGF-βRII and is phosphorylated, stimulating disassembly of the signalling complex and thereby inhibiting TGF-β signalling. Endoglin also promotes signalling through the ALK2-SMAD1 pathway, which inhibits cell migration.

Protein kinase CK2

Protein kinase CK2 (formerly casein kinase 2) is a ubiquitously expressed serine/threonine kinase with many intracellular targets and roles in a variety of processes [49]. CK2 has been shown to phosphorylate PRH at residues S163 and S177 within its homeodomain (**Fig. 4, red arrows**), inhibiting DNA binding by PRH and targeting it for proteasomal cleavage [50,51]. Cleavage by the proteasome produces a truncated PRH protein, termed PRHΔC, that acts as a transdominant negative regulator of the full length protein by sequestering TLE co-repressors, and possibly other PRH-binding proteins [51]. Aberrantly high CK2 expression or activity is thought to be oncogenic in a number of cancers, due to inactivation of several tumour suppressors including PML and PTEN [52]. CK2 has been shown to be more active in benign prostatic hyperplasia (BPH) and prostatic adenocarcinoma [53]. In line with this, it was recently found that PRH is hyperphosphorylated in BPH and prostate cancers, and this phosphorylation is dependent upon CK2 [54]. Furthermore, overexpression of CK2 abrogates the inhibition of cell migration and proliferation by PRH in prostate cancer cells [54]. CK2 could therefore represent a viable drug target in disease states promoted by PRH deficiency resulting from CK2 overactivity [55]. For example, it is known that increased Src kinase activity in many cancers increases CK2 activity; the BCR-ABL/Src kinase inhibitor Dasatinib has been shown to inhibit proliferation of leukaemic cells via increased PRH-dependent repression of Vegf and Vegfr-1 [56].

TGF-β signalling may regulate PRH phosphorylation as part of a feedback loop

The ability of PRH to upregulate the TGF-βRIII Endoglin has been well studied, while Endoglin is known to modulate pro-EMT ALK5 signalling [17,36,48]. Additionally, PRH is known to be phosphorylated by protein kinase CK2, a modification blocking its DNA binding activity and marking it for proteasomal cleavage [50,54]. It is therefore possible that cellular responses to TGF-β may be dictated by the phosphorylation status of PRH. Indeed, PRH is hyper-phosphorylated by CK2 in prostate cancer cells and breast cancer cells [54]. Interestingly, it has recently been observed that TGF-β treatment results in a decrease in abundance of PRH and a concomitant increase in phosphorylated PRH (Eudmar Marcolino, unpublished observations). This suggests that there exists an auto-regulatory feedback loop between TGF-β signalling and PRH activity, possibly mediated by CK2. If so, CK2 would represent a valuable drug target in metastasis and disease states supported by CK2-driven PRH deficiency.

Project Aims

This project therefore seeks to elucidate how TGF-β and platelets may influence prostate cell motility and EMT status, and to ascertain if this is reinforced by TGF-β-dependent changes in PRH phosphorylation and abundance.

Specifically, the project aims to:

- Assess the impact of TGF-β and platelets upon prostate cell migration using chemotaxis assays.
- Measure any TGF-β or platelet-induced changes in EMT marker expression, both at the protein level using immunoblotting and at the level of transcription using quantitative PCR.
- Study how PRH levels affect TGF-β-mediated EMT signalling using adenoviral overexpression of PRH
- Determine if TGF-β signalling alters the phosphorylation status of PRH, and if this is facilitated by protein kinase CK2 using a specific inhibitor its kinase activity.

Materials and Methods

Cell Culture

The immortalised 'normal' prostate cell line PNT2-C2, originally established by transfection of primary culture with SV40 genome, was obtained from Professor Norman J Maitland (University of York). PC3 cells, representing an aggressive metastatic prostate cancer cell type, were obtained from the ATCC (Manassas, VA, USA). These cell lines were selected because although immortalised PNT2-C2 are no longer strictly comparable to normal physiological prostate cells (they lack expression of p53, for example), they are a good approximation of normal epithelial cell behaviour when compared to mesenchymal PC3 cancer cells. Hence, comparison of cellular responses by PNT2-C2 and PC3 cells represents a convenient model for comparing normal and cancerous prostate responses to stimuli.

Cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 2mML-glutamine and 1% penicillin/streptomycin (herein Complete Media, CM) and maintained in a humidified atmosphere at 37 °C and 5% CO_2. When cells were to undergo treatment, they were seeded at $1.5x10^5$ cells per well of a 6-well Cellstar® plate (Greiner Bio-One, Frickenhausen, Germany) in triplicate. The following day, media was removed and replaced with media containing either 5ng/ml TGF-β1 (PeproTech, Cat#100-21C) or platelets (kindly provided by the Alistair Poole lab group, University of Bristol) in a 1:1 ratio with plated cells. Where stated, cell were also incubated with the ALK5 inhibitor SB-431542 (Sigma, S4317) at 3μM or the specific CK2 kinase inhibitor CX-4945 (Silmitasertib, Abcam) at various concentrations. Cells were then incubated in the above conditions for 48 hours, unless stated otherwise.

When comparing platelet fractions, the 'supernatant' was prepared by centrifuging an untreated platelet suspension at 10,000rpm in an Eppendorf Centrifuge 5415 R for 10 min and then adding 1.25μl of this supernatant to every 10ml of CM to be added to cells. 'Releasate' was prepared by mixing the total number of platelets required for incubation with PC3 cells in a 3:1 ratio, adding 1ml CM and incubating at 37°C for 30 min. This was centrifuged at 10,000rpm for 10 min, the supernatant retained and made up to the total volume required for incubation in CM. 'Media supernatant' was prepared by incubating platelets in CM alone at 37°C for 30 min. When treating cells with buffer alone, 1.25μl was added to every 10ml of CM required.

Cell Migration Assays

Following treatment chemotaxis assays were performed by seeding cells into 8 μm Boyden chambers (Greiner Bio-One, Frickenhausen, Germany) in RPMI-1640 with 2% fetal bovine serum. The chambers were placed into 24-well plates containing RPMI-1640 with 10% fetal bovine serum

to create a serum gradient. Cells were incubated in a humidified atmosphere at 37 °C and 5% CO_2 and allowed to migrate for 12 hours.

Initially, after migration the wells were submerged in 1X 0.5% Trypsin-EDTA (Gibco) for 5 min so that migrated cells would detach into the Trypsin-EDTA. These cells were pelleted by centrifugation at 1,000rpm for 5 minutes, fixed with 4% paraformaldehyde (Fisher), stained with 3 µM DAPI (4',6-diamidino-2-phenylindole) in staining buffer (0.1M Tris, 0.15M NaCl, 1mM $CaCl_2$, 0.5mM $MgCl_2$, 0.1% Triton). Fluorescence was then quantified in a FLEXstation plate reader (Molecular Devices, Sunnyvale, CA, USA) using SOFTmax PRO 3.2 (Molecular Devices, San Diego, CA, USA) with excitation at 358nm and emission read at 461nm. In order to derive a cell count from raw fluorescence values, a standard curve of fluorescence from a serial dilution of a known number of cells was produced.

In later iterations, after migration the RPMI-1640 media containing 10% FBS in the bottom of the plate was replaced with RPMI-1640 containing 8µM Calcein-AM (Calbiochem, EMD Millipore Corp., Billerica, MA USA) and incubated for 45 min in a cell culture incubator at 37°C and 5% CO_2. The transwells were then moved into wells containing 500µl pre-warmed Trypsin-EDTA and incubated at 37°C and 5% CO_2 for 10 minutes, with frequent agitation. The transwells were discarded and 200µl of the Trypsin-EDTA, now containing migrated cells, was transferred into a black 96-well plate. Fluorescence was then quantified in a FLEXstation plate reader using SOFTmax PRO 3.2 at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. In order to derive a cell count from raw fluorescence values, a standard curve of fluorescence from a serial dilution of a known number of cells was produced.

Western Blot Analysis

Following incubation, cells were harvested and replicates pooled. The cells were pelleted by centrifugation at 11,000rpm for 5 min in an Eppendorf Centrifuge 5415 R. This pellet was then resuspended in 1X Cell Lysis Buffer (#9803 Cell Signalling, Danvers, MA, USA) with 1mM PMSF protease inhibitor (#8553 Cell Signalling, Danvers, MA, USA) and 2X PhosSTOP phosphatase inhibitor (Roche, #04906845001). Samples underwent SDS-PAGE on 8% polyacrylamide gels and were incubated with primary and then secondary antibodies at the dilutions and times indicated below. Densitometric analysis was performed using Image Studio Lite Version 5.2 (LiCor).

Antibodies

Antibody target	Dilution used	Diluent (5% in TBS-T)	Incubation time	Clone	Species	Supplier	Code
β-actin	1:5000	Milk	1 hour	Monoclonal	Rabbit	Cell Signalling (Danvers, MA, USA)	13E5
E-cadherin	1:1000	Milk	Overnight				24E10
Snail	1:1000	Milk	Overnight				C15D3
pSMAD3	1:1000	Milk	Overnight				C25A9
PRH (M6)	1:100,000	Milk	Overnight	Monoclonal	Mouse	Produced in-house	-
pPRH (YKN5)	1:2,500	BSA	Overnight	Monoclonal	Rabbit		-
PRH	1:1000	Milk	Overnight	Monoclonal	Mouse	Origene (Rockville, MD, USA)	OTI4B7
Lamin A/C	1:5000	Milk	1 hour	Polyclonal	Rabbit	Santa-Cruz (Dallas, TX, USA)	sc-20681
Rabbit IgG	1:5000	Milk	1 hour	-	Goat		sc-2301
Mouse IgG		Milk	1 hour				sc-2055

Quantitative PCR

Following incubation, cells were harvested and replicates pooled. The cells were pelleted by centrifuging at 11,000rpm for 5 min in an Eppendorf Centrifuge 5415 R. Total RNA was then isolated using a Qiagen RNeasy Kit, following the manufacturer's instructions. cDNA was then immediately synthesised using a Qiagen QuantiNova Reverse Transcription Kit, following the manufacturer's instructions. Quantitative PCR was then performed in technical quadruplicates with E-cadherin, PRH and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) primers (shown below), all at 1μM, in a Qiagen Rotor-Gene Q. GAPDH mRNA was used as an internal control. Data were analysed using Q-Rex software.

E-cadherin 5'-GTAACGACGTTGCACCAACC-3', 5'-AGCCAGCTTCTTGAAGCGAT-3'

PRH 5'-AGCTCTCAATGTTCGCCCTC-3', 5'-TCGCCCTCAATGTCCACTTC-3'

GAPDH 5'-TGATGACATCAAGAAGGTGGTGAAG-3'
5'-TCCTTGGAGGCCATGTGGGCCAT-3'

Overexpression of myc-tagged PRH

A recombinant adenoviral construct expressing Myc-PRH, as described by Soufi et al [57], was used. Cells were suspended and incubated in CM containing 100 virus particles per cell at 37°C and 5% CO_2 for 5 minutes. The cells were then plated into a 6-well plate at an initial seed of $1.5x10^5$ cells per well. A control group of cells incubated with an adenoviral construct expressing

GFP were plated in parallel. After 24 hours, the media was replaced with CM containing no supplement, 5ng/ml TGF-β1 or platelets at a 5:1 ratio to cells.

Statistical analysis

All statistical analyses was performed in GraphPad Prism 6.

Results

TGF-β increases migration in a SMAD-dependent manner in PNT2-C2 cells and PC3 cells

TGF-β signalling is known to be make a crucial contribution to aberrant EMT in some cancers, and platelet-derived TGF-β has been shown to be particularly important in promoting metastasis in breast and colorectal carcinoma cells that enter the vasculature [5,30,58]. The nature of TGF-β signalling was studied using the small molecule inhibitor SB-431542, which inhibits phosphorylation of SMAD2/3 by ALK4, ALK5 and ALK7 [59]. Importantly, SB-431542 does not inhibit other kinase signalling pathways, including those activated by TGF-β receptors independent of the canonical SMAD pathway.

PNT2-C2 ‘normal’ immortalised prostate cells and PC3 prostate cancer cells were incubated for 48 hours with either no supplement or isolated TGF-β, with and without SB-431542. Cells were then allowed to migrate through Boyden chambers under a serum gradient, with the number of migrated cells quantified using the fluorescent dye Calcein-AM. Cell migration is expressed as fold change relative to untreated cells without SB-431542. In both cell lines pre-incubation with TGF-β increases cell migration around 2-fold, indicating that these cells lines respond to TGF-β with pro-EMT signalling and an increase in cell motility (**Fig. 6**). Co-incubation with SB-431542 largely blocks this increase suggesting that that the observed increase in cell migration is, at least partly, mediated by ALK5 and signalling via SMAD proteins. However, when the data presented in **Figure 6b** underwent two-way ANOVA and no statistically significant differences were observed when the cell migration of ‘No Treatment, No Inhibitor’ cells was compared to all other treatment conditions. The observation that TGF-β increases cell migration should therefore be treated with caution. Additionally, it must be noted that the data presented in **Fig. 6a** only represents n=2, and are therefore only preliminary. This assay must therefore be repeated in both cell lines to lend the above observations validity and significance.

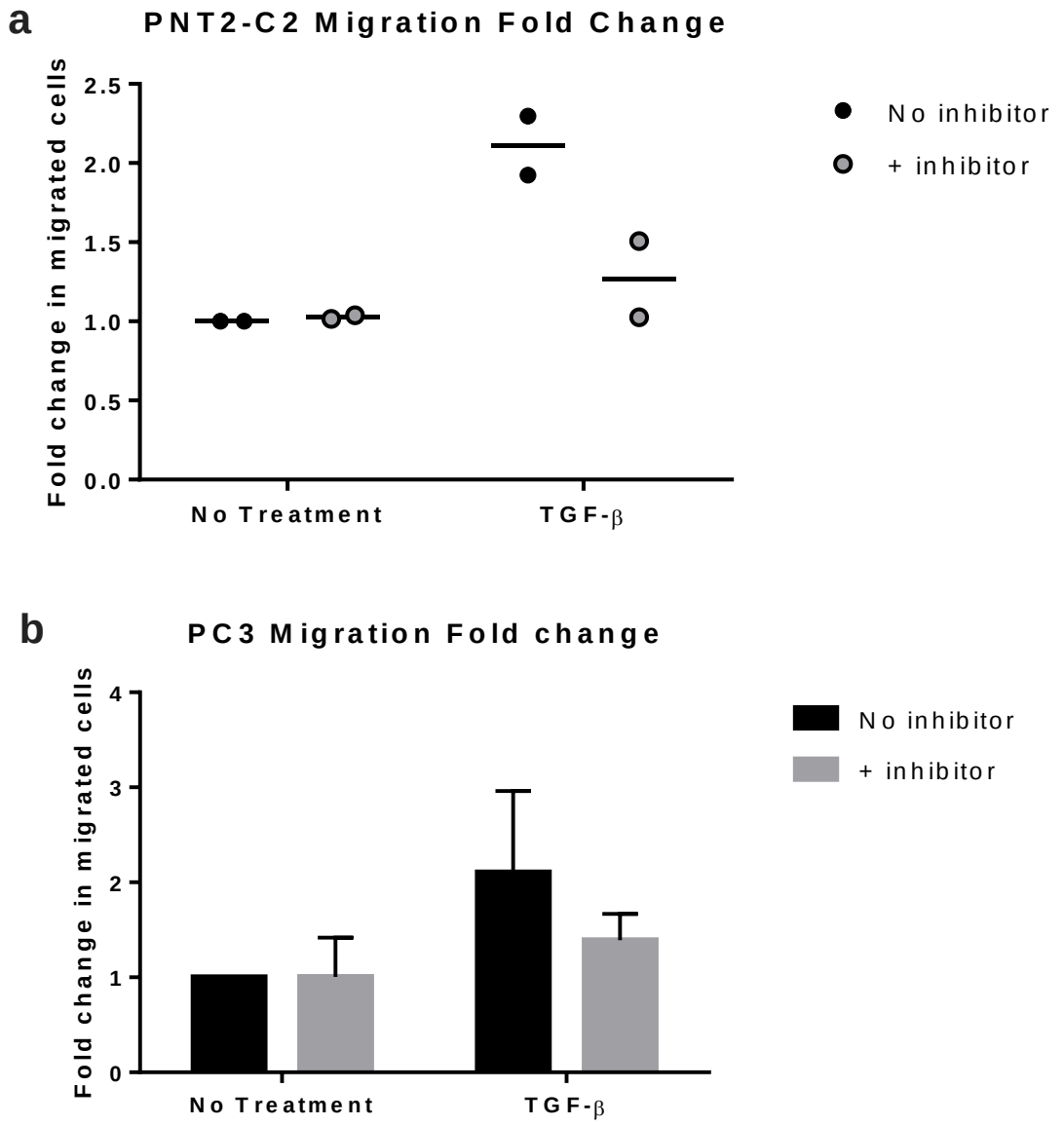


Figure 6. The ALK5 inhibitor SB-431542 blocks TGF-β-stimulated increase in cell migration

Cells were incubated in CM with no supplement or isolated TGF-β, with or without SB-431542. Cells were harvested and then seeded into Boyden chambers under a serum gradient and allowed to migrate for 12 hours. The high serum media was then replaced with RPMI-1640 containing 8µM Calcein-AM for 45 minutes. The transwells were moved into Trypsin-EDTA for 10 minutes, with agitation. 200µl of the Trypsin-EDTA, containing migrated cells, was transferred to a black 96-well plate and fluorescence measured at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. Migration is expressed as fold change relative to untreated cells without the inhibitor SB-431542. **Figure a** represents n=2 with individual data points shown, black bar represents the mean. **Figure b** is n=3, error bars represent SD.

b) 'No Treatment, No Inhibitor' cells were compared to all other treatment conditions in two-way ANOVA with Dunnett's multiple comparison test:

'No Treatment + Inhibitor' p>0.99, 'TGF-β, No Inhibitor' p=0.06, 'TGF-β + Inhibitor' p=0.67.

TGF-β downregulates E-cadherin and PRH in PNT2-C2 cells

Further to studying the effect of TGF-β treatment upon prostate cell migration, changes in key protein markers were examined. PNT2-C2 were incubated for 48 hours with either no supplement or isolated TGF-β. Cells were then harvested and lysates underwent SDS-PAGE and Western blotting. The cell-cell adhesion molecule E-cadherin is a key marker for EMT, being a final target and convergence point of several pro-EMT signalling pathways [5,58,60]. Incubation with TGF-β corresponded with a decrease in detectable E-cadherin in PNT2-C2 cells, potentially reflecting pro-EMT signalling in these cells (**Fig. 7a**).

PRH can negatively regulate TGF-β signalling in prostate cells via its ability to upregulate Endoglin, an auxiliary type III TGF-β receptor [36,46]. It has been proposed that TGF-β signalling may negatively regulate PRH, mediated by phosphorylation by protein kinase CK2, creating an auto-regulatory feedback loop. In support of this, it was found that incubation with TGF-β resulted in a marked decrease in PRH levels in PNT2-C2 cells; PRH protein expression was reduced to levels comparable to those of a CCLP1 cell line in which PRH has been knocked down (**Fig. 7b**). It should be noted these observations are preliminary and only represent n=1, and should therefore be repeated to validate the above observations.

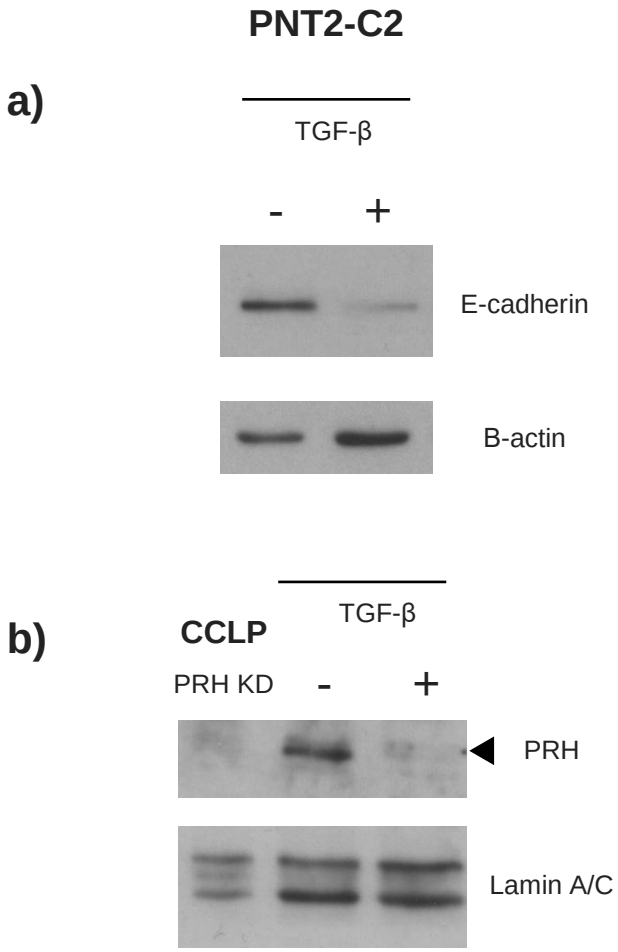


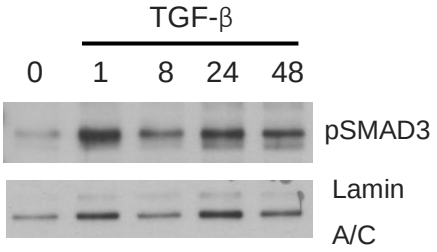
Figure 7. Incubation with TGF-β reduces E-cadherin and PRH in PNT2-C2 cells

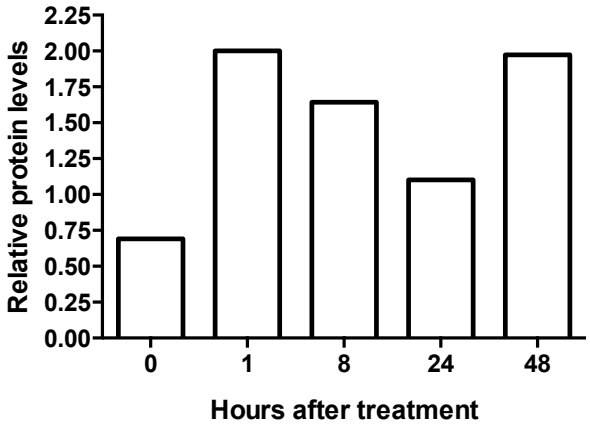
PNT2-C2 cells were incubated in CM for 48 hours, either with or without 5ng/ml TGF-β. When blotting for PRH, a PRH-knockdown CCLP cell line was used as a negative control (**b**, leftmost lane). Cells were lysed and proteins underwent SDS-PAGE. Lamin A/C was used as a loading control for PRH, while β-actin was used as a loading control for E-cadherin. n=1.

TGF-β downregulates E-cadherin and upregulates pSMAD3 and Snail in PC3 cells

To characterise the effect of TGF-β upon prostate cancer cells, PC3 cells were treated with TGF-β and the changes in key protein markers over time was quantified using Western blotting and densitometric analysis. The changes in phospho-SMAD3, Snail and E-cadherin were examined before treatment (time = 0) and then at 1, 8, 24 and 48 hours after treatment. Snail is a transcription factor upregulated by pro-EMT signalling that, among other functions, binds to the *CDH1* gene encoding E-cadherin and represses its transcription [5]. The addition of TGF-β results in a rapid increase in pSMAD3 levels, corresponding to signalling through the ALK5-SMAD pathway. pSMAD3 levels remain higher than in untreated cells for up to 48 hours (**Fig. 8a**). In contrast to the data presented in **Figure 7a**, here E-cadherin levels decrease during incubation with TGF-β (**Fig. 8b,** experimental repeats are presented separately). In the first iteration presented here (**Fig. 8b #1**) E-cadherin levels are markedly decreased at 1 hour of incubation and fall to their lowest levels at 24 hours, with decrease appearing to have plateaued or begun to reverse by 48 hours. In the second iteration (**Fig. 8b #2**), E-cadherin levels do not decrease until 24 hours, reaching lowest the point at 48 hours. Snail levels increase dramatically at 8 hours and plateau by 24 hours of incubation with TGF-β (**Fig. 8c**). It is interesting to note that during sustained TGF-β signalling pSMAD3 levels appeared to decrease after their peak at 1 hour of incubation, while levels of E-cadherin and Snail continued to change up to at least 24 hours. This is consistent with findings in human keratinocytes that acute TGF-β signalling induces a refractory period in receptor-mediated SMAD activation, which is independent of any downstream signalling already set in motion [61]. It also suggests that during chronic exposure to TGF-β, SMAD-independent signalling pathways may be important for enacting pro-EMT signalling in prostate cancer cells. However, these interpretations are at best speculative since the data presented here is only n=1 and n=2, and so will require repetition to confirm these observations. Indeed, the difference in time for E-cadherin levels to decrease in **Fig. 8b #1** and **#2** indicates potentially substantial variability in the cellular responses. Repetition may be particularly important regarding the blots relating to pSMAD3, as repetition may confirm or eliminate the apparent cyclical activation.

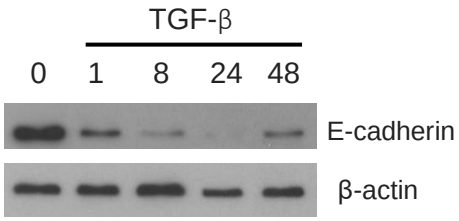
a) pSMAD3

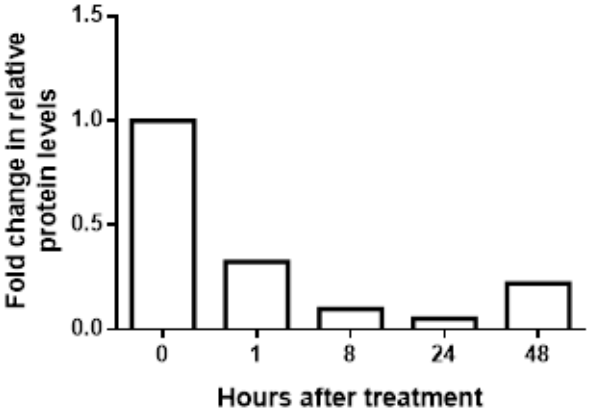




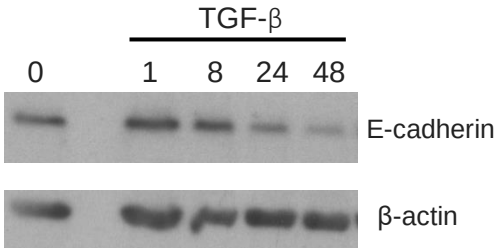
b) E-cadherin

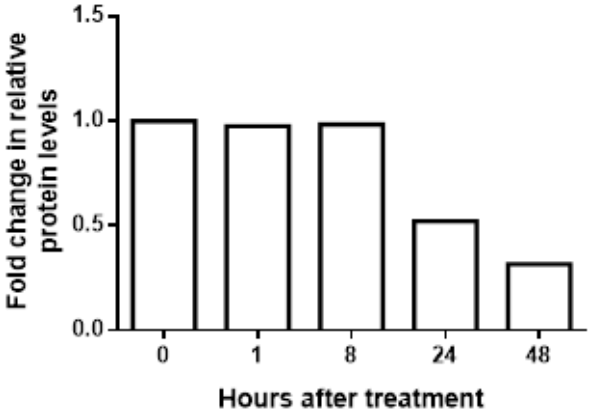
#1



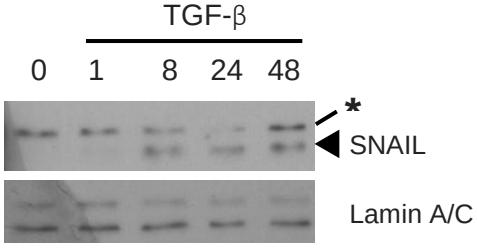


#2





c) Snail



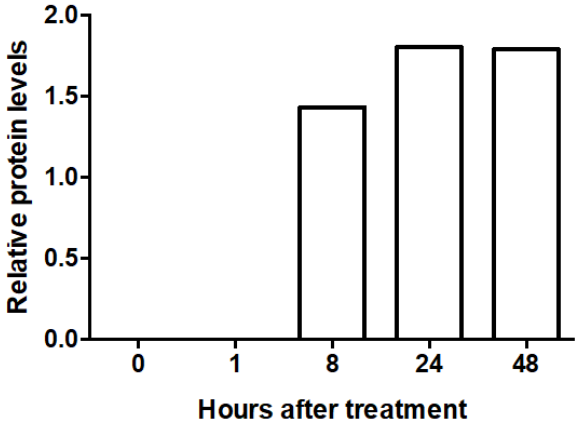


Figure Legend below.

Figure 8. In PC3 cells, after the addition of TGF-β pSMAD3 levels peak at 1 hour but E-cadherin and Snail levels continue to change until at least 24 hours

PC3 cells were incubated in CM for 1, 8, 24 or 48 hours with 5ng/ml TGF-β. Time 0 corresponds to untreated cells harvested when other samples were treated. Cells were lysed and proteins underwent SDS-PAGE, and membranes were probed with antibodies for phosho-SMAD3 (**a**), E-cadherin (**b**) or Snail (**c**). Films were developed and bands were analysed by densitometry. n=1 for blots of pSMAD3 and SNAIL. n=2 for E-cadherin, with both blots and corresponding densitometry shown (**#1 and #2**). Lamin A/C was used as a loading control for pSMAD3 and Snail, while β-actin was used as a loading control for E-cadherin. * indicates non-specific antibody binding.

PRH overexpression in prostate cancer cells blocks EMT signalling by TGF-β

Phosphorylation of PRH by the protein kinase CK2 blocks DNA binding by PRH and marks it for cleavage by the proteasome [43]. It has been shown that PRH is hyperphosphorylated in in BPH, prostatic adenocarcinoma and prostate cancers, and this phosphorylation is dependent upon CK2 [54]. Since levels of functional PRH are reduced in prostate cancers, myc-tagged PRH was overexpressed in PC3 cells using an adenoviral vector and the cells were then treated with isolated TGF-β. This allows us to study the relationship between PRH levels and EMT signalling in the context of the prostate cancer intracellular environment. Separate cells were transfected with an expression vector for GFP, in place of a scrambled control vector. E-cadherin levels were used as readout of EMT status. The myc-tag was also probed for, to gauge the success of PRH overexpression, but the myc-tagged PRH was not detected during immunoblotting (data not shown). Incubation with TGF-β reduced E-cadherin levels in cells not incubated with adenovirus by nearly 80% (**Fig. 9a**), in line with expected resulting pro-EMT signalling and that seen in **Figure 8b** [5,30]. Transfection with the myc-PRH vector causes a mean 3.5-fold increase in E-cadherin levels (**Fig. 9b**). In these cells, treatment with TGF-β caused some reduction in E-cadherin, but levels remained a mean of 2.4-fold higher than untreated cells. Importantly, among cells incubated with TGF-β those that were infected with the myc-PRH vector had levels of E-cadherin 11-fold higher than untransfected cells (**Fig. 9**, second and sixth bars from the left, respectively). This suggests that in the prostate cancer intracellular environment, in which endogenous PRH is hyper-phosphorylated, increased levels of PRH are capable of blocking pro-EMT signalling by TGF-β.

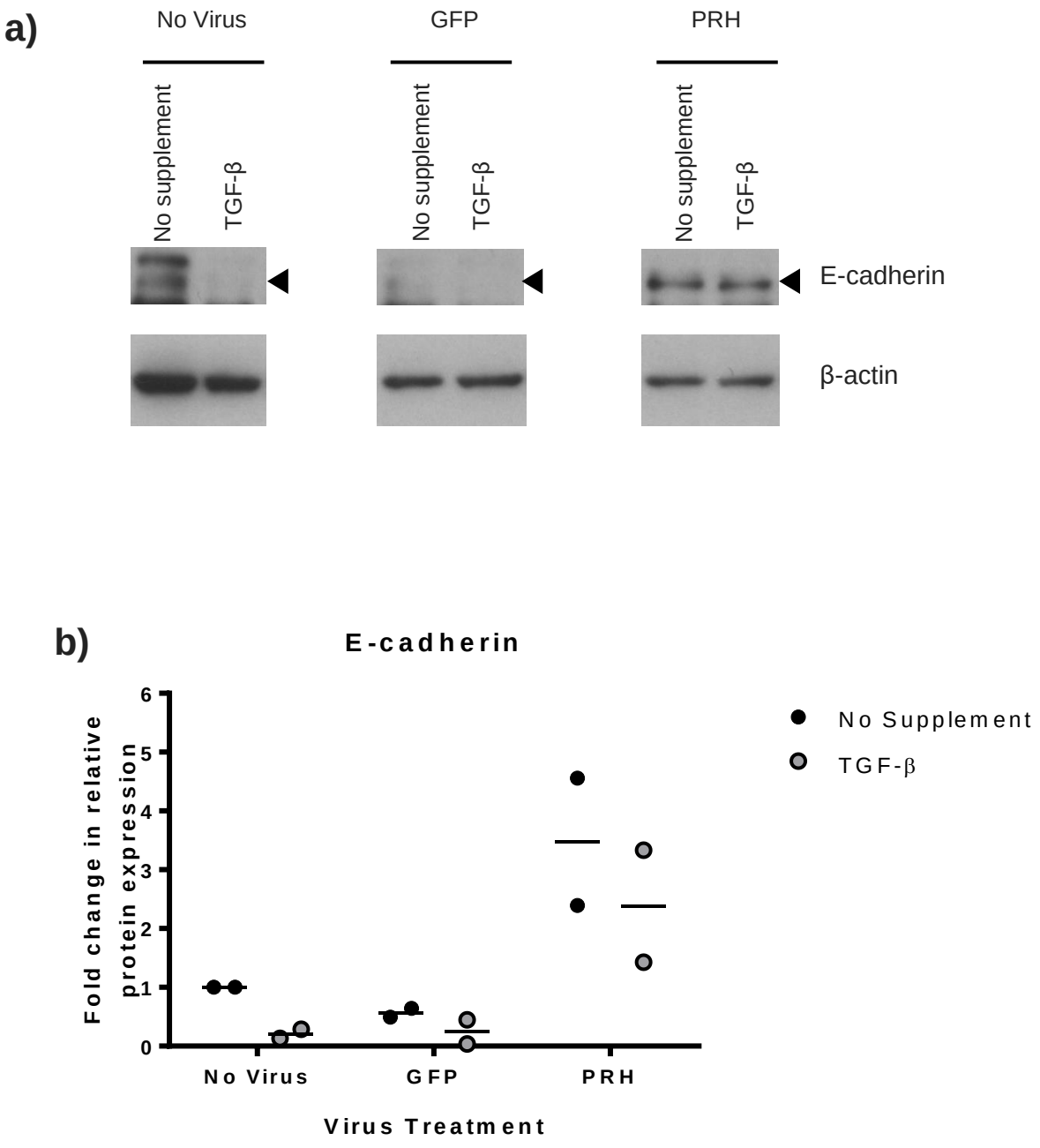


Figure 9. Overexpression of PRH in PC3 cells blocks TGF-β-stimulated decrease in E-cadherin levels

A recombinant adenoviral construct expressing Myc-PRH or GFP was used. Cells were suspended and incubated in CM containing 100 virus particles per cell for 5 minutes and then were then plated and incubated for 24 hours. Media was then replaced with CM containing no supplement or 5ng/ml TGF-β and incubated for 48 hours. Cells were then harvested and lysed. Following immunoblotting and film development, bands were analysed by densitometry (n=2). Individual data points are presented, horizontal bar represents the mean. β-actin was used as a loading control for E-cadherin.

Treatment with TGF-β appears to raise levels of both total PRH and phospho-PRH in a CK2-independent manner in PC3 cells

While PRH is known to modulate TGF-β signalling through its regulation of the TGF-βRIII Endoglin, it has been suggested that PRH is itself negatively regulated by TGF-β. The proposed regulation of PRH is thought to be mediated by the protein kinase CK2, which phosphorylates PRH and marks it for proteasomal processing [50,51]. This would be demonstrated by a CK2 inhibitor blocking any TGF-β-induced reduction of PRH or increase in phospho-PRH. PC3 cells were incubated with TGF-β and increasing concentrations of the CK2 inhibitor CX-4945 (Silmitasertib) for 48 hours, with the intention of establishing what concentration of the inhibitor was necessary to block changes in PRH or phospho-PRH (pPRH) levels. Although IC_{50} for CX-4945 is 1nM, concentrations up to 20μM were tested because 5ng/ml of TGF-β is a saturating concentration that could promote relatively high levels of signalling through CK2 [62]. All cells incubated with TGF-β had levels of PRH above that of untreated cells, regardless of coincubation with CX-4945 of any concentration (**Fig 10a**). From this data it is difficult to identify a trend in PRH levels in relation to CX-4945 concentration, particularly since the data is only n=1. Probing for phospho-PRH hints at the existence of the proposed phenomenon; incubation with TGF-β increases levels of pPRH above that of untreated cells (**Fig. 10b**). It must be noted that although the Western blot bands for pPRH do not appear to change between incubation conditions, analysis by densitomentry and normalisation of this signal to the bands for the loading control Lamin A/C indicates that all cells incubated with TGF-β exhibit higher levels of pPRH than the untreated cells. The TGF-β-mediated increase in pPRH appears at least partly blocked by 2μM and 5μM CX-4945. However, this is confounded by levels of pPRH rising at higher concentrations of inhibitor. Taken together, these data do not indicate that CX-4945 has any clear influence on any TGF-β-mediated changes in PRH or pPRH levels. However, these data only represent n=1 and should therefore be treated as preliminary.

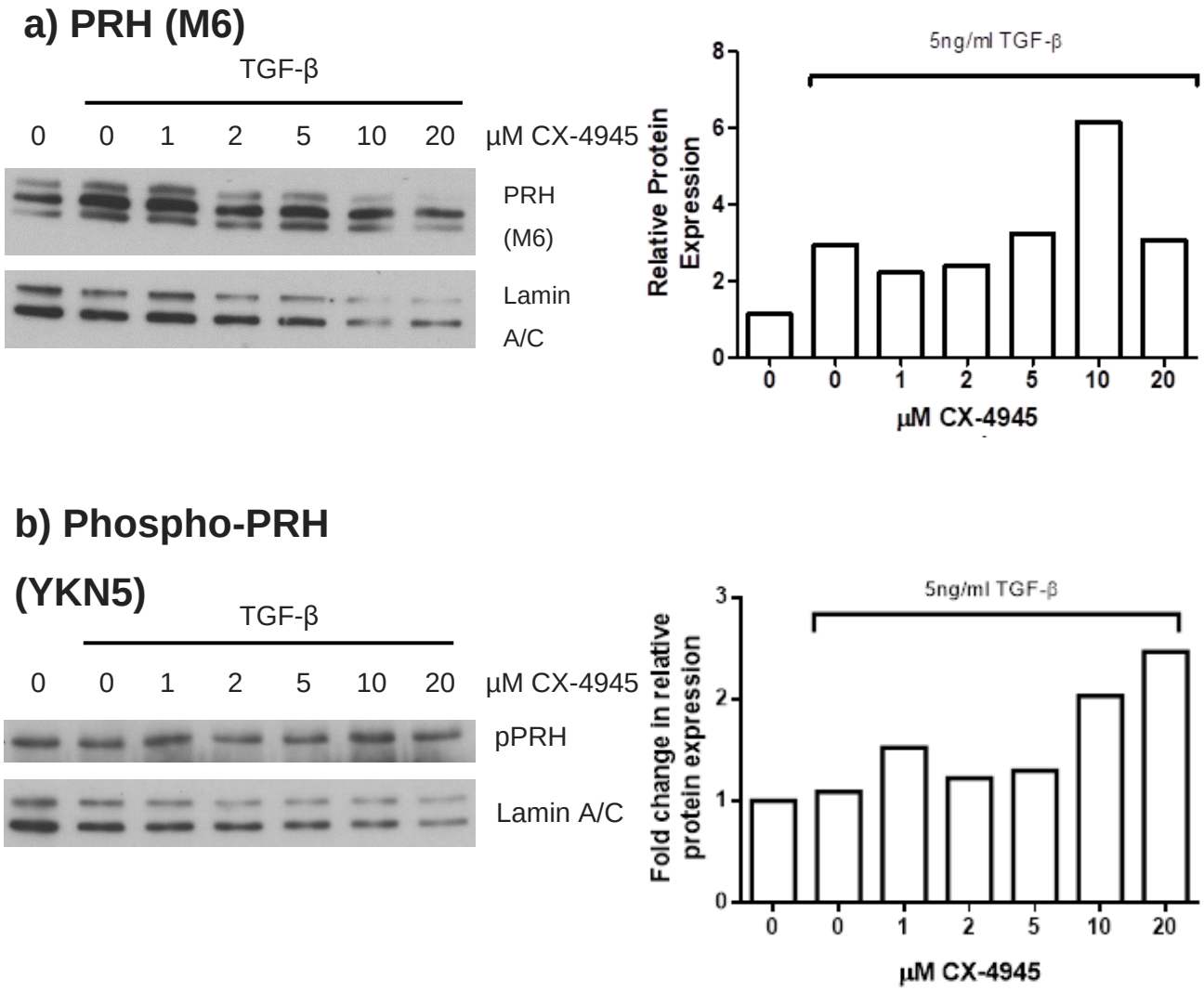


Figure 10. TGF-β-induced increases in pPRH are partially blocked by CX-4945 at 2µM and 5µM, but not higher doses

PC3 cells were incubated in CM or CM supplemented with 5ng/ml TGF-β and 0, 1, 2, 5, 10 or 20µM CX-4945 for 48 hours. Cells were lysed, underwent SDS-PAGE and were probed using an antibody for PRH (M6, **a**) or phosphorylated PRH (YKN5, **b**). Following immunoblotting and film development, bands were analysed by densitometry (n=1). Lamin A/C was used as the loading control.

Platelets increase cell migration in both normal prostate and prostate cancer cells

Platelets are a prominent store of TGF-β in the body, and accordingly may represent a physiologically important source of TGF-β signalling for metastatic cells that reach the vasculature [28–30]. Platelets have been shown to promote EMT and the migration of breast and colorectal carcinoma cells, and have been proposed to reinforce the mesenchymal phenotype and thereby enable circulating cancer cells to extravasate and reach a secondary niche [30]. Since this is mediated by a combination of platelet-derived TGF-β signalling and contact dependent signalling, incubation of prostate cells with platelets could result in similar or greater increases in cell migration to those observed when treated with TGF-β alone, as in **Figure 6**. To investigate this, PNT2-C2 cells and PC3 cells were incubated in a 1:1 ratio with platelets for 48 hours. The cells were then allowed to migrate through a porous membrane from low to high serum content for 12 hours. Preliminary data, gathered by quantifying the total number of cells that migrate though the membrane using a fluorimeter, confirm the expected result that prostate cancer cells migrate faster than normal cells (**Fig. 11**). Furthermore, the data indicates that pre-incubation with platelets considerably increases cell motility in PNT2-C2 cells, and to a lesser degree in PC3 cells.

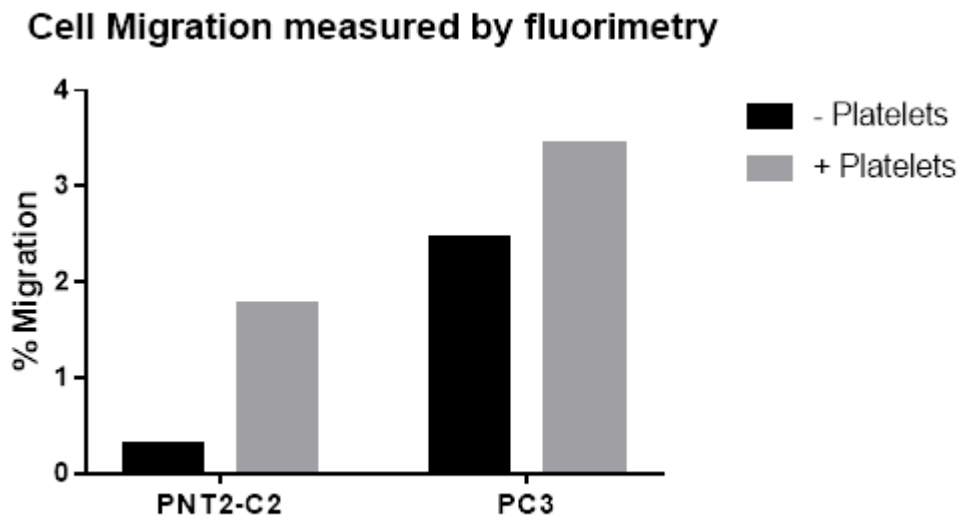


Figure 11. Platelets increase cell migration in normal and prostate cancer cells

Cells were incubated in CM with or without a 1:1 ratio of platelets for 48 hours, and then seeded into Boyden chambers under a serum gradient and allowed to migrate for 12 hours. All of the cells that had migrated through were collected, fixed with 4% paraformaldehyde, stained with DAPI and fluorescence was measured. The total number of migrated cells was calculated and expressed as a percentage of the total seeded into the transwell (n=1)

Platelet buffer constituents do not promote EMT signalling

It has been shown in breast and colon carcinoma cells that incubation with platelets promotes EMT signalling and cell migration to a greater degree than treatment with platelet releasate alone [30]. To recapitulate these findings in prostate cell lines would be to confirm that any observed changes in cell migration or protein expression are a result of the platelet interaction with prostate cells in the work presented here. PNT2-C2 and PC3 cells were incubated with either complete media (CM) alone ('no treatment'), platelets in a 5:1 ratio of platelets to cells, the equivalent volume of the 'supernatant' fraction of a non-activated platelet suspension, or the releasate from platelets activated by cancer cells. E-cadherin was used as the readout for any resulting EMT signalling, since it is a key marker at the convergence of several signalling pathways [5]. In the preliminary data presented here, incubation with platelets caused a reduction in E-cadherin levels in PC3 cells but not in PNT2 cells (**Fig. 12**). Incubation with the 'supernatant' fraction, corresponding to suspension buffer after platelets have been pelleted and removed, did not cause reduction in E-cadherin levels. This suggests that any observed pro-EMT signalling is neither a result of constituents of the buffer, nor any factors passively released by platelets prior to their interaction with cells. Unexpectedly, the 'supernatant' fraction appeared to cause an increase in E-cadherin in PNT2-C2 cells. As with platelet incubation, the addition of releasate from activated platelets caused a reduction in E-cadherin levels in PC3 cells but not PNT2-C2 cells. This platelet releasate-mediated reduction was smaller than that caused by the addition of platelets, supporting the notion that platelets exert their effects via contact-dependant signalling and release of factor-filled granules [30].

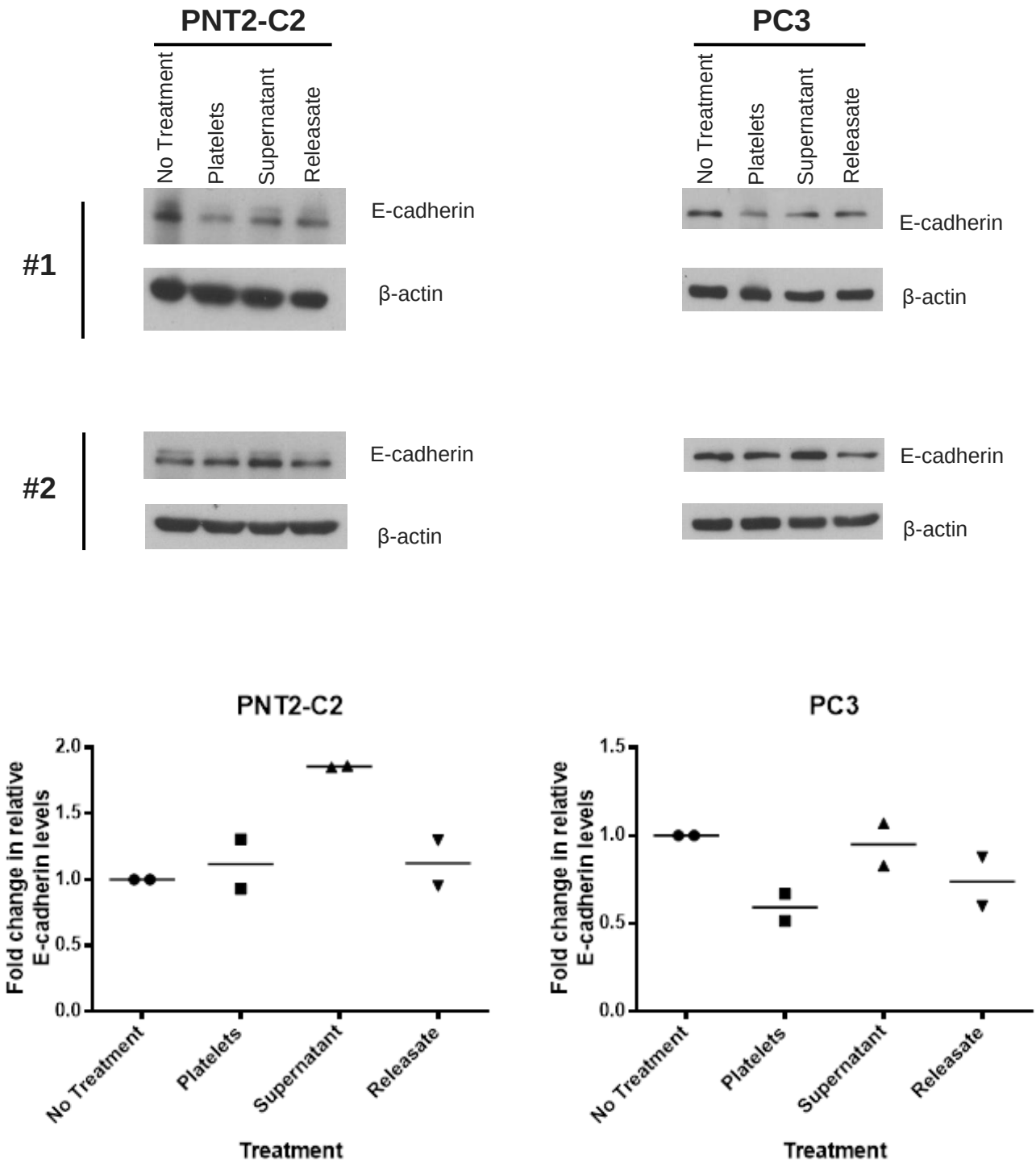


Figure 12. Platelet suspension 'supernatant' fails to mimic E-cadherin loss induced by platelets

Prostate cell lines were incubated for 48 hours with CM containing no supplement, platelets, platelet 'supernatant', or platelet releasate. Samples were harvested, underwent SDS-PAGE and were Western blotted for E-cadherin levels. N=2, with both iterations of Western Blotting shown (**#1 and #2**). Bands were analysed by densitometry, using β-actin as a loading control.

Platelets fail to replicate TGF-β-mediated decreases in E-cadherin in PNT2-C2 and PC3, while TGF-β increases PRH levels in PC3 cells

Notwithstanding the established role of TGF-β in inducing EMT and cell motility, platelets can influence cancer cell behaviour through direct contact and the secretion of myriad other factors [30,63]. In order to establish the importance of TGF-β signalling in this, the ALK5 inhibitor SB-431542 was used to assess the contribution of TGF-β signalling to any effect platelet contact had upon EMT marker protein levels. PNT2-C2 and PC3 cells were incubated for 48 hours with either no supplement, isolated TGF-β or with platelets in a 5:1 ratio, each with or without SB-431542. Cells were harvested and lysed, and proteins underwent SDS-PAGE. Western blots were probed for the key EMT marker E-cadherin, PRH and in one case Snail, a pro-EMT transcription factor. In PNT2-C2 cells TGF-β treatment results in a reduction in E-cadherin levels (**Fig. 13a**). Although the Western blot shown here suggests a reduction in E-cadherin when PNT2-C2 cells are incubated with platelets, densitometric analysis and normalisation to the loading control β-actin reveals that platelet incubation had no apparent effect on E-cadherin levels. In contrast to the data presented in **Figure 7**, in PNT2-C2 cells TGF-β treatment appeared to cause no change in PRH levels (**Fig. 13b**). However, these data only represents n=1 and therefore require repetition before any meanful conclusions can be drawn.

Similar to PNT2-C2 cells, PC3 cells incubated with TGF-β exhibited a decrease in E-cadherin and an increase in Snail levels – corresponding to pro-EMT signalling – that was not replicated by incubation with platelets (**Fig. 14a and b**, respectively). Densitometric analysis reveals that PRH levels were consistently increased by incubation with TGF-β yet SB-431542 did not block this increase (**Fig. 14c**), indicating that this increase may be mediated by TGF-β-activated signalling pathway other than the canonical SMAD pathway. Platelets appeared to promote a slight decrease in PRH levels in PC3 cells but coincubation with SB-431542 caused levels to rise above those of untreated cells, making the relationship between platelets, TGF-β signalling and regulation of PRH unclear. It should be noted that the data presented in **Figures 14a** and **14c** did not achieve statistical significance when analysed in two-way ANOVA comparing ‘No Supplement –SB-431542’ to all other treatment conditions. Therefore, all differences of protein levels when compared to untreated cells should be treated as preliminary observations.

It is worth noting that the inability of platelets to promote reduction of E-cadherin in PC3 cells in these experiments contrasts the data presented in **Figure 12**, where incubation with platelets resulted in a notable reduction of PC3 E-cadherin levels. This points to variable efficacy of platelet incubations.

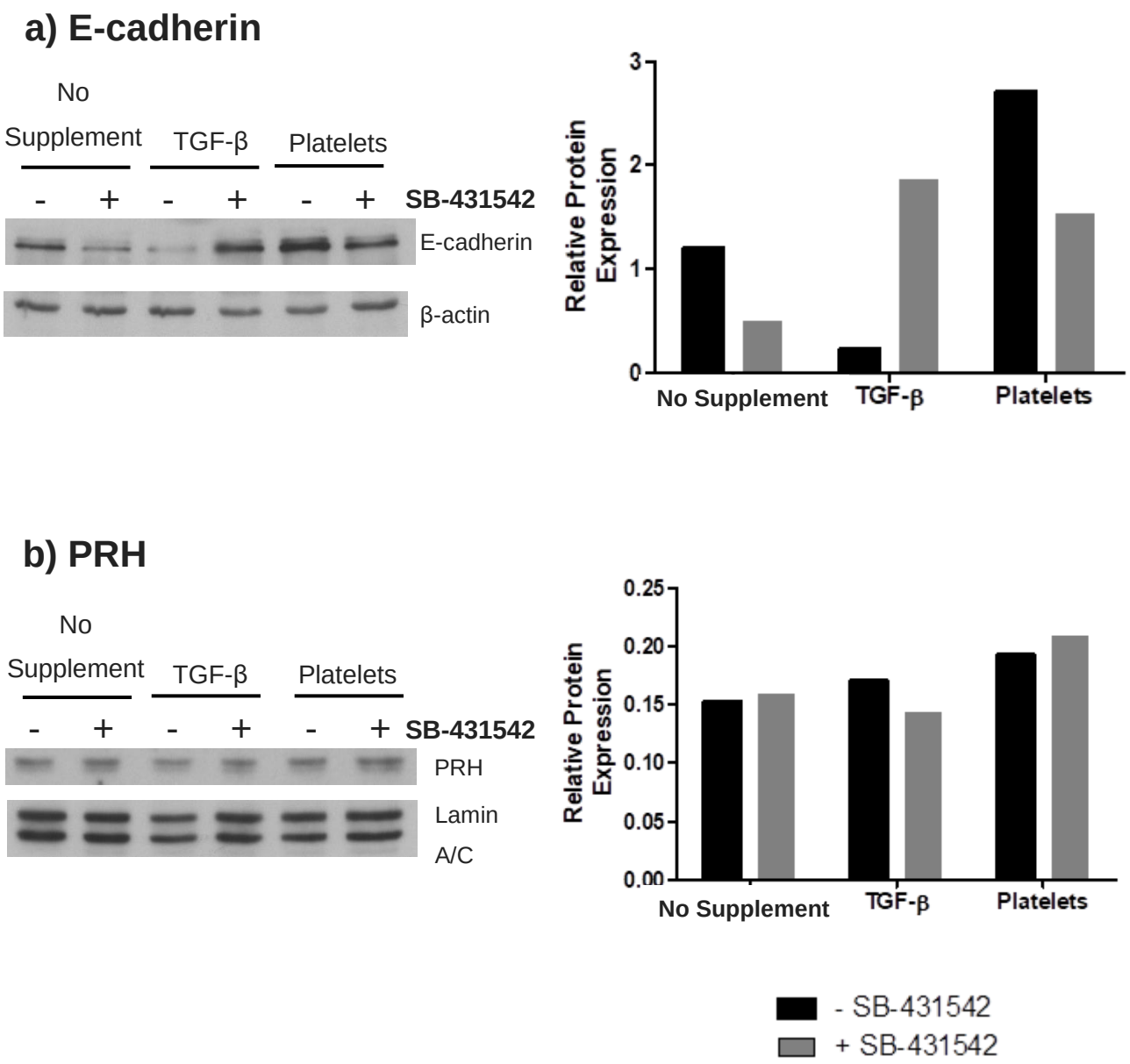


Figure 13. In PNT2-C2 cells platelets do not replicate the reduction in E-cadherin levels caused by isolated TGF-β, while neither treatment caused a significant change in PRH levels

PNT2-C2 cells were incubated in CM with no supplement, isolated TGF-β or with platelets in a 5:1 ratio, with or without SB-431542. Cells were lysed and underwent SDS-PAGE. Lamin A/C was used as a loading control for PRH and Snail, while β-actin was used for E-cadherin. Blots presented are representative examples (left). Blots were analysed by line densitometry, normalising to loading controls (right). Where protein levels are expressed as fold change, it is relative to the untreated samples (leftmost bar).

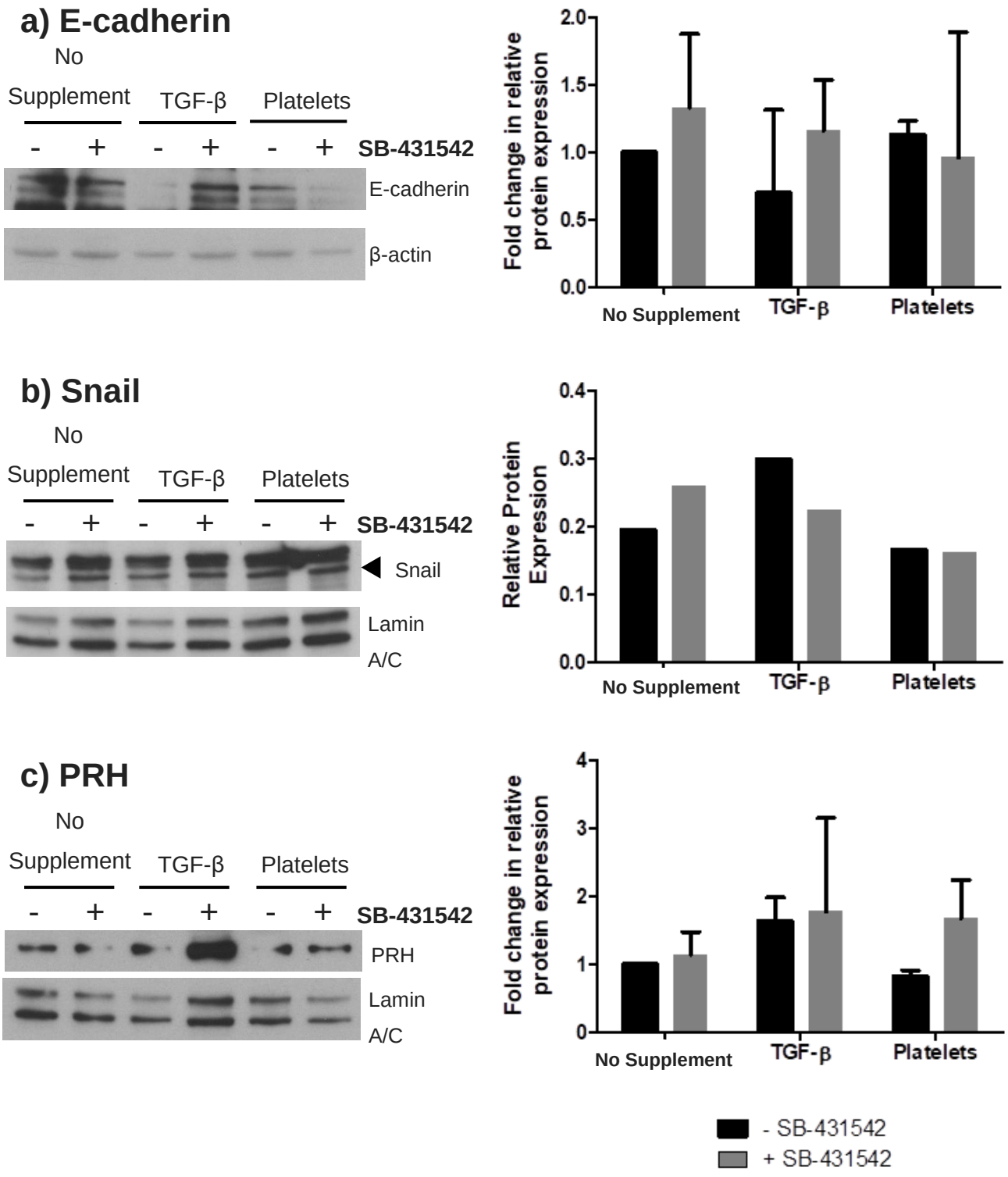


Figure Legend below.

Figure 14. In PC3 cells platelets do not replicate changes in E-cadherin, Snail and PRH levels caused by isolated TGF-β

PC3 cells were incubated in CM with no supplement, isolated TGF-β or with platelets in a 5:1 ratio, with or without SB-431542. Cells were lysed and underwent SDS-PAGE. Lamin A/C was used as a loading control for PRH and Snail, while β-actin was used for E-cadherin. Blots presented are representative examples (left). Blots were analysed by line densitometry, normalising to loading controls (right). Where protein levels are expressed as fold change, it is relative to the untreated samples (leftmost bar). Where error bars are presented, error bars correspond to standard deviation and n=3 (**a and c**). n=1 for **b**

For data in **a** and **c** 'No Supplement, -SB-431542' cells were compared to all other treatment conditions in two-way ANOVA with Dunnett's multiple comparison test:
a) 'No Supplement, +SB-431542' p=0.67, 'TGF-β -SB-431542' p=0.80, 'TGF-β +SB-431542' p>0.99, 'Platelets -SB-431542' p=0.99, 'Platelets +SB-431542' p=0.99.
b) 'No Supplement, +SB-431542' p=0.97, 'TGF-β -SB-431542' p=0.77, 'TGF-β +SB-431542' p=0.41, 'Platelets -SB-431542' p>0.99, 'Platelets +SB-431542' p=0.75.

TGF-β promotes *E-cadherin* mRNA decrease in PNT2-C2 and PC3 cells, and platelets decrease *E-cadherin* mRNA in PC3 cells

Further to studying the effect of platelet incubation on cell migration and protein markers, E-cadherin and PRH mRNA levels were examined and compared with those of cells incubated with TGF-β. PNT2-C2 and PC3 cells were incubated for 48 hours with either no supplement, isolated TGF-β or with platelets in a 5:1 ratio. mRNA was isolated from cell lysates and cDNA created, which underwent quantitative PCR (qPCR) with primers for E-cadherin or PRH, with GAPDH (glyceraldehyde-3-phosphate dehydrogenase) used as an internal control. qPCR was performed in technical quadruplicate, with mean values presented here. In PNT2-C2 cells, incubation with TGF-β results in a slight decrease in *E-cadherin* mRNA levels while platelets cause an apparent increase (**Fig. 15a**), in line with changes in E-cadherin protein levels in **Figure 13**. In PC3 cells levels of *E-cadherin* mRNA are reduced around 2-fold following incubation with TGF-β (**Fig. 15b**). *E-cadherin* mRNA was reduced approximately 4-fold when incubated with platelets, indicating greater EMT signalling. However, this contrasts the inability of platelets to promote reduction of E-cadherin protein in **Figure 14a**, suggesting that platelet-mediated effects are not consistently translated into protein-level changes within 48 hours. However, this interpretation is based on data which only represents n=1 and should be treated as preliminary until validated by repetition.

TGF-β does not decrease PRH mRNA levels in PNT2-C2 or PC3 cells

In addition, *PRH* mRNA levels were studied following the treatment described above. In PNT2-C2 cells, *PRH* mRNA does not appear to be affected by incubation with platelets. Incubation with TGF-β caused an apparent 16-fold increase in *PRH* mRNA levels, although a change of this magnitude may reflect an experimental error (**Fig. 16a**). Incubation with TGF-β has little or no effect upon *PRH* mRNA levels in PC3 cells, while platelets appear to cause an approximate 2-fold reduction (**Fig. 16b**). This would appear to contrast data presented in **Figure 14c**, where measurements at the protein level indicated that TGF-β promotes increased levels of cellular PRH. It is worth noting that all of these data are limited in that they are only n=1, and should therefore be treated with caution.

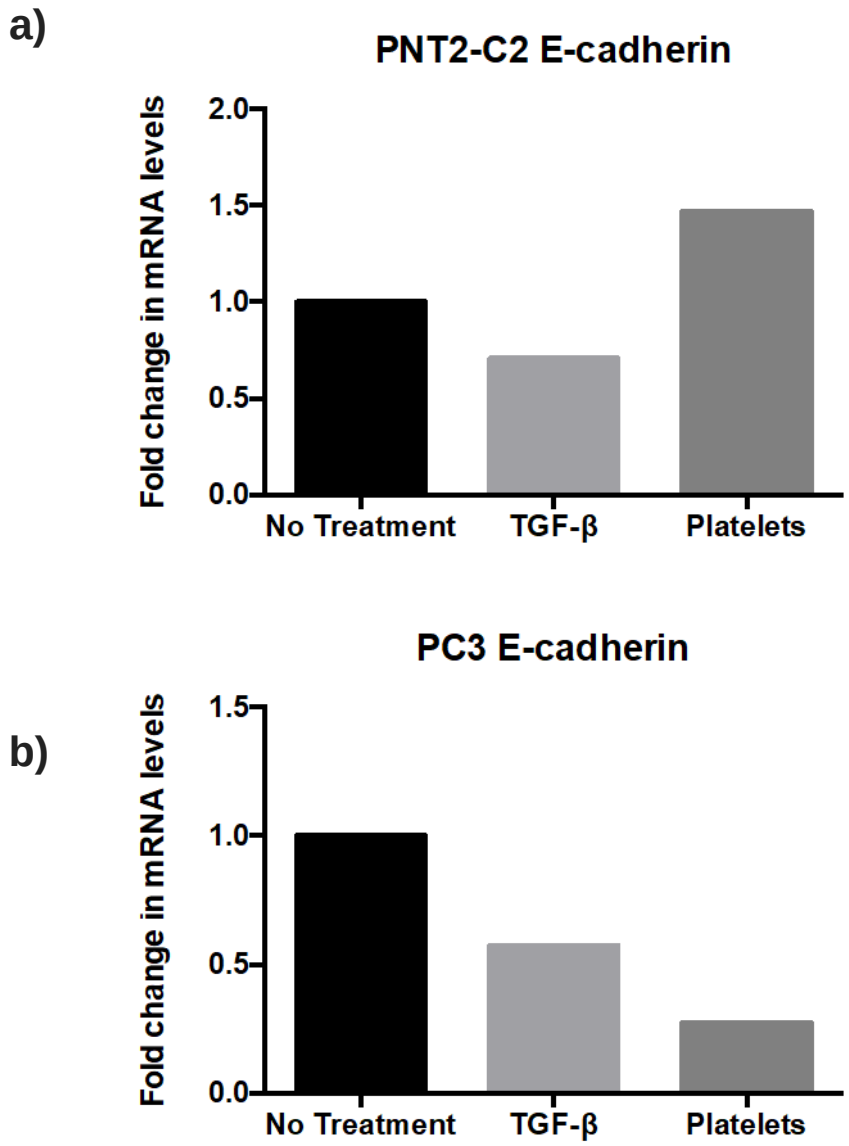


Figure 15. TGF-β promotes decrease in E-cadherin mRNA in PNT2-C2 and PC3 cells, while platelets only reduce E-cadherin in PC3 cells

PNT2-C2 and PC3 cells were incubated in CM with no supplement, isolated TGF-β or with platelets in a 5:1 ratio. Total RNA was isolated using a Qiagen RNeasy Kit and cDNA was then immediately synthesised using a Qiagen QuantiNova Reverse Transcription Kit, both following the manufacturer's instructions. Quantitative PCR was then performed in a Qiagen Rotor-Gene Q in technical quadruplicates, with mean values presented here. GAPDH mRNA was used as an internal control. Data were analysed using Q-Rex software. n=1.

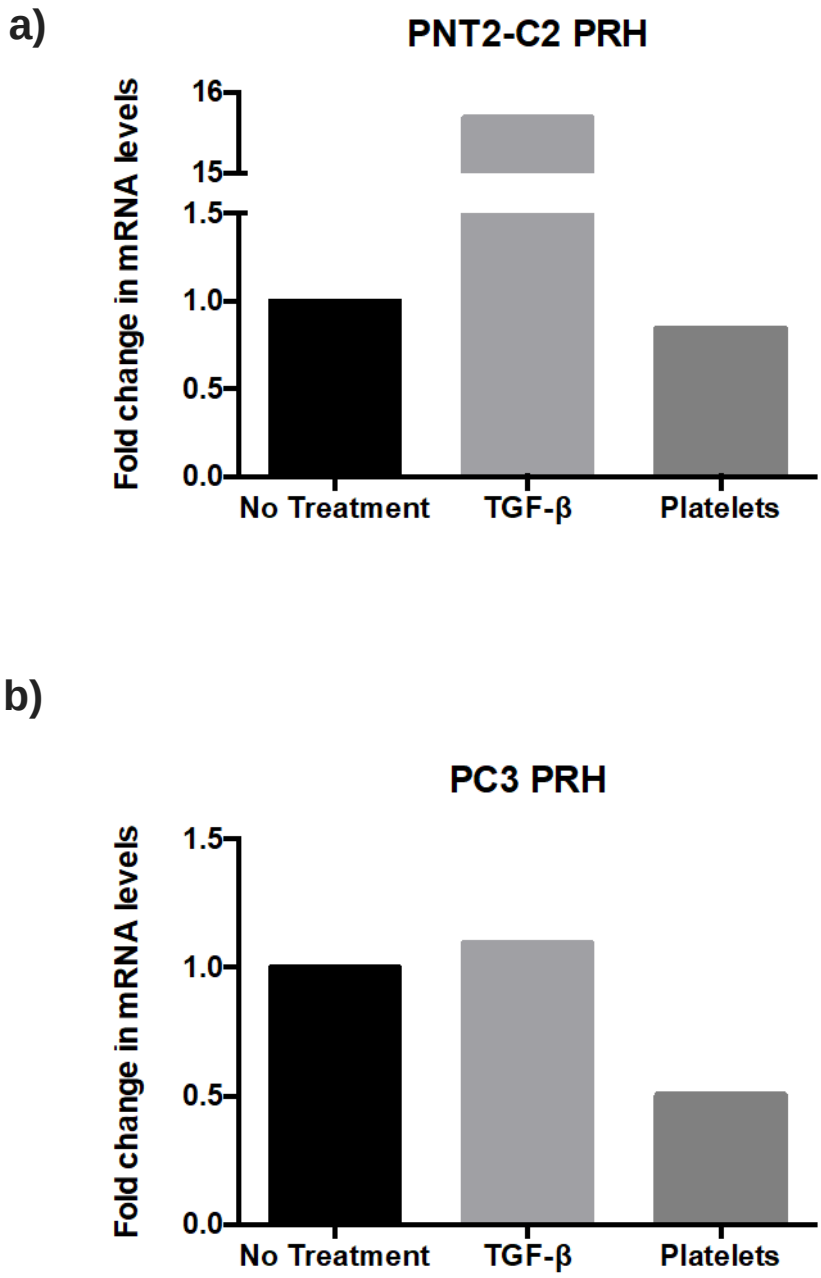


Figure 16. TGF-β has very little effect on PRH mRNA in either PNT2-C2 or PC3 cells, while incubation of platelets almost halves PRH levels in PC3 cells

PC3 cells were incubated in CM with no supplement, isolated TGF-β or with platelets in a 5:1 ratio. Total RNA was isolated using a Qiagen RNeasy Kit and cDNA was then immediately synthesised using a Qiagen QuantiNova Reverse Transcription Kit, both following the manufacturer's instructions. Quantitative PCR was then performed in a Qiagen Rotor-Gene Q in technical quadruplicates, with mean values presented here. GAPDH mRNA was used as an internal control. Data were analysed using Q-Rex software. n=1.

Discussion

Via its direct regulation of factors including Goosecoid, VEGF and Cyclin D1, and its modulation of TGF-β signalling via Endoglin, PRH is a key regulator of EMT [43]. PRH dysregulation is therefore potentially capable of promoting aberrant EMT and acquisition of a migratory phenotype in adult cells, enabling metastasis of cancer cells. In prostate cancer cells and breast cancer cells it has been shown that PRH is hyperphosphorylated, and that this is dependent upon the protein kinase CK2 [54]. Since phosphorylated PRH is unable to bind DNA and is marked for cleavage, it is less able to upregulate Endoglin to antagonise TGF-β signalling. In this manner, it is thought that pro-EMT TGF-β signalling may occur without an important restraint in prostate and breast cancer cells. Furthermore, it has been proposed that TGF-β signalling causes a reduction in cellular PRH levels. Platelets are a particularly rich physiological source of TGF-β, and it has been demonstrated that platelet-derived TGF-β enhances metastasis of breast and colon carcinomas in mice [30]. The above work represents an investigation into the ability of TGF-β and platelets to promote EMT and migration in normal prostate and prostate cancer cell lines. Most importantly, this project aimed to elucidate how PRH influences, and is influenced by, these processes.

Pre-incubation with TGF-β increased cell migration in immortalised ‘normal’ prostate and prostate cancer cell lines, in concordance with several cancers and cell types [30,58,60]. That SB-431542 blocked this increase indicates that in prostate cell lines TGF-β-mediated cell migration is largely mediated by the canonical SMAD effector proteins. However, these data do not exclude the involvement of SMAD-independent pathways such as Erk-MAP kinase, JNK or PI3K pathways [5], and future work assessing how their contribution might combine with that of TGF-β would no doubt be informative. For example, this could represent useful information in the development of therapeutic strategies that target multiple pathways to inhibit prostate cancer cell migration. The preliminary data presented here also suggests that platelets are likewise capable of increasing cell migration in both normal prostate and prostate cancer cells by promoting pro-EMT phenotypic changes.

E-cadherin was used extensively as a readout of EMT status, since it is regulated at the final convergence of several pro-EMT signalling pathways [5,58,60]. In several experiments it was found that incubation with platelets did not mimic reductions in E-cadherin caused by isolated TGF-β in either cell line. Despite platelets not causing a reduction in E-cadherin at the protein level, there was preliminary evidence that in PC3 cells *E-cadherin* mRNA levels were reduced by platelets to a greater degree than by TGF-β. This suggested that during a 48 hour incubation changes in E-cadherin gene transcription have occurred that had not yet been translated through to the protein level. However, this interpretation is based upon a single iteration of qPCR and is at best speculative, with further experiments needed for confirmation. The qPCR data presented in

Figures 15 and 16 represent the culmination of several rounds of unsuccessful experimental attempts and subsequent troubleshooting, where qPCR results were rejected due to unacceptable variance in technical repeats (threshold cycle values greater than 1 cycle apart) and unacceptably high amplification in non-template controls. With these issues apparently resolved to within acceptable limits, repetition of the experiments may now validate the preliminary observations made here.

In the experiments described involving SB-431542, platelets consistently did not replicate the reduction in E-cadherin caused by incubation with TGF-β. This contrasts the earlier observation that platelets produced a pro-EMT response of E-cadherin decrease during work comparing the effect of incubation with platelets, buffer 'supernatant' or platelet releasate. The disparity in the efficacy of platelet treatment suggests that variation among the platelets used is a crucial determinant of experimental success. Indeed, the platelets are donated on the morning of their use by volunteers who may be of diverse genetic backgrounds and lifestyles. They may also be collected and prepared by various people.

It has been shown that PRH is hyper-phosphorylated and thereby downregulated in prostate cancers. Therefore, an aim of the work presented here was to compare the response of the PC3 prostate cancer cell line to TGF-β to that of cells in which the status of the PRH population is normal – here, the immortalised 'normal' prostate cell line PNT2-C2. However, there are a multitude of differences in their intracellular environments which may confound this comparison. Therefore, myc-tagged PRH was adenovirally overexpressed in PC3 cells in order to compare responses between cancer cells in which the only difference was PRH expression levels. Overexpression of PRH caused a greater than three-fold increase in E-cadherin levels. This is consistent with previous work in which PRH overexpression inhibits cell migration and invasion by prostate and breast cancer cell lines, and supports work in which PRH knockdown reduces levels of E-cadherin in normal prostate cells [36,54]. The preliminary findings presented here may therefore extend the current body of evidence for the role of PRH as a negative regulator of EMT in prostate cells. Although treatment of these cells with TGF-β caused a reduction in E-cadherin, its levels remained around 2-fold higher than untreated cells. This demonstrates that increased levels of PRH may be capable of modulating TGF-β signalling and EMT in PC3 cells, an aggressive cancer cell line of high metastatic potential. Therefore, a therapeutic agent that raises cellular PRH levels may represent an effective inhibitor of metastasis, at least in prostate cancers.

It has been proposed that TGF-β signalling causes a reduction in PRH levels via CK2-mediated phosphorylation. Here, a 48-hour incubation with TGF-β gave a mixed response in PNT2-C2 cells; a marked decrease in one experiment and little change in another. PC3 cells, on the other hand, respond to TGF-β with an increase in PRH levels wherever PRH is detectable by immunoblotting.

As predicted, incubation with TGF-β resulted in an apparent increase in pPRH in PC3 cells. An increase in both PRH and pPRH levels was an intriguing observation because phosphorylation of PRH marks it for proteasomal cleavage, and thus changes in their abundance is expected to be reciprocal. It is possible that while TGF-β treatment does result in an increase in PRH phosphorylation events, it also results in an upregulation in total PRH levels in PC3 cells (**Fig. 17, right**). Indeed, while it has been previously demonstrated that pPRH is more abundant in prostate cancer cell lines than normal prostate cells, their total hypo-phosphorylated PRH remains comparable [54]. Such a scenario might point to a rapid shutoff mechanism, in which increased phosphorylation of PRH serves to rapidly downregulate PRH levels upon cessation of TGF-β signalling (**Fig. 17, right, red arrow**). Alternatively, the increase in detected pPRH upon TGF-β stimulation may simply be a consequence of an upregulation of total PRH, with the proportion of the population that is phosphorylated remaining constant. It will be interesting to determine which of these processes actually occurs, and future work on this question might make use of an inducible, non-phosphorylatible form of PRH. Importantly, in PC3 cells neither the observed changes in PRH nor pPRH were blocked by CX-4945, contesting the hypothesis that TGF-β signalling regulates PRH via the protein kinase CK2 (**Fig. 17**). It is also possible that PNT2-C2 and PC3 cells – representing non-cancerous and cancerous populations, respectively – react to TGF-β with distinct signalling pathways: The preliminary data here indicates that in PNT2-C2 cells PRH is downregulated, potentially via CK2, while PC3 cells appear to upregulate PRH through a currently unknown pathway. Should these findings be substantiated, future work may seek to elucidate how PRH is upregulated in PC3 cells and identify any kinases responsible for phosphorylation of PRH. An inhibitor of these kinases may represent valuable therapeutic agents in the prostate cancer cell where PRH is upregulated but hyperphosphorylated, because dephosphorylation of this population may enable inhibition of pro-EMT TGF-β signalling via upregulation of Endoglin.

PC3 cells display a hyper-phosphorylated PRH population compared to PNT2-C2 cells [54]. Both isolated TGF-β and platelets are capable of increasing cell migration in PNT2-C2 and PC3 cells, suggesting that the differing statuses of PRH populations in these cells does little to influence their responses to pro-EMT signalling via TGF-β. However, the contrasting changes in PRH protein levels in these cell lines suggests that the relationship between TGF-β signalling and PRH may be more complex than previously thought. Rising levels of PRH in PC3 cells after incubation with TGF-β point to a possible negative feedback loop, whereby the increased PRH negatively regulates TGF-β signalling via upregulation of the TGF-βRIII Endoglin. It is difficult to assess if this phenomenon is specific to the cancer intracellular environment, however, due to a paucity of data from the normal prostate cell line PNT2-C2. This was unfortunately due to persistent and recurring bacterial contaminations of the PNT2-C2 cells being used in the laboratory, which was eventually discovered to be due to contamination of the stock cells stored in liquid nitrogen. Future work in

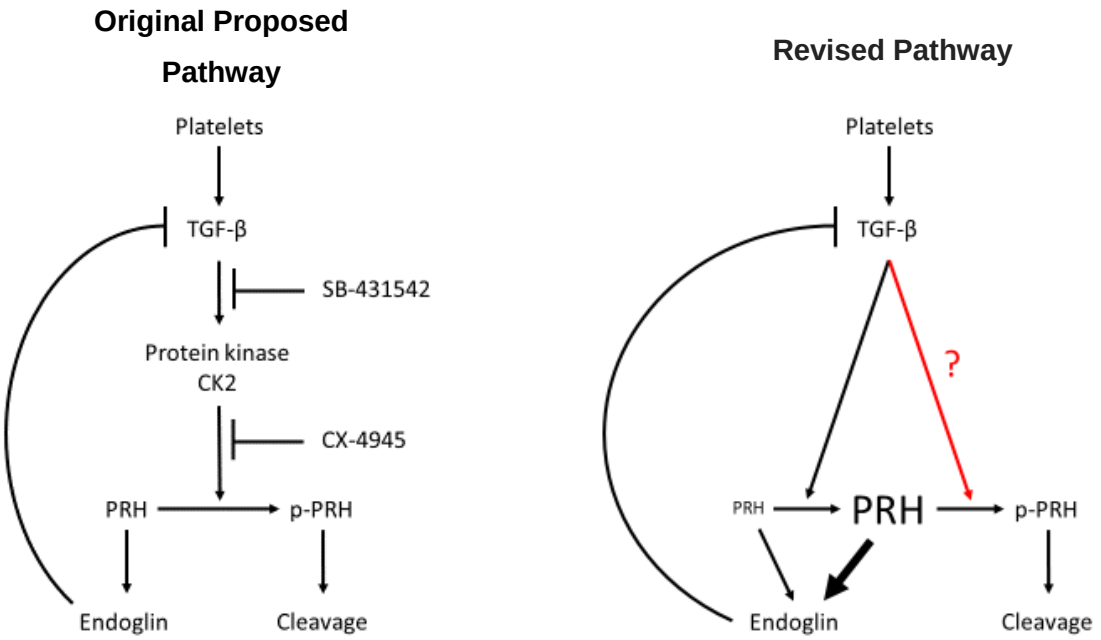


Figure 17. Diagrams depicting the original proposed signalling pathway, and a preliminary revised pathway based on experimental observations

Right) The originally proposed signalling pathway. Platelet derived TGF-β would signal through Protein Kinase CK2 to phosphorylate PRH and mark it for degradation. Downregulation of PRH would subsequently downregulate expression of Endoglin, thereby removing an inhibitor of TGF-β signalling. Notably, this signalling pathway may be representative of PNT2-C2 responses to TGF-β.
Left) A revised signalling pathway for PC3 cell responses to TGF-β, based on the preliminary data presented here. TGF-β signalling instead promotes an increase in cellular PRH levels, although the signalling intermediates remain unknown. Increased PRH levels would potentially increase expression of Endoglin, thereby reinforcing the negative feedback loop upon TGF-β signalling. The postulated increase in PRH phosphorylation mediated by TGF-β is shown in **red**, as it is unclear from the data presented here whether it is a real consequence of increased kinase activity or simply a consequence of an increase in total cellular PRH.

this area will undoubtedly require repetition of these investigations in PNT2-C2 in much greater numbers, and potentially in other cell lines of known PRH status.

Additional further study may focus on the influence on PRH on cellular processes besides its modulation of TGF-β via endoglin. As mentioned previously, PRH is capable of inhibiting the activity of the proto-oncogene c-myc by blocking its interaction with its obligate DNA binding

partner, Max [45]. Future work may probe how any TGF-β-mediated changes in PRH abundance and phosphorylation alter the activity of c-myc, since dysregulation of c-myc is one of the most frequently observed changes in cancers. Indeed, during this project attempts were made to utilise fluorescent immunostaining to study how nuclear abundance and colocalization of PRH and c-myc in prostate cell lines might change with TGF-β stimulation, but the procedure could not be satisfactorily optimised in the time available. If successful, fixing and staining cells at several time points may have revealed if PRH levels changed in response to TGF-β in the nucleus, cytoplasm or entire cell in PNT2-C2 and PC3 cells, and the timescale on which these changes occurred. Such data might be informative in determining the timeframes in which the function of nuclear c-myc may be perturbed by TGF-β-induced changes, revealing the dynamics of how the interactions between PRH and c-myc may contribute to oncogenesis.

Taken together, these preliminary observations suggest that the story surrounding PRH and EMT may be more complex than previously thought. While upregulation of PRH appears to block TGF-β-mediated EMT in PC3 prostate cancer cells, and thus may represent a strategy to oppose metastasis, how TGF-β in turn affects PRH may be more multifaceted. PRH appears to be upregulated by TGF-β in the prostate cancer cell line but may instead be downregulated in the comparatively ‘normal’ PNT2-C2 cell line, suggesting that TGF-β-mediated changes in PRH may be altered by certain cancer-related cellular alterations. This may be of importance because based on their intracellular environments prostate cells might react very differently to blockade of TGF-β signalling during therapy. For example, inhibition of the signalling pathway that upregulates PRH in response to TGF-β in PC3 cells may prevent a concomitant upregulation of Endoglin and thereby derepress total TGF-β signalling, leading to an increase in signalling through other TGF-β-activated pathways. Diverse therapeutic outcomes of TGF-β-blockade may therefore be possible based on the response of PRH, and so study of this transcription factor’s behaviour in varying cellular contexts may be of vital importance when designing personalised and targeted therapeutic strategies for prevention of metastasis in prostate cancer.

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